REMARKS

Introductory Comments

Claims 1, 3, 5-7, 11-14 and 19-21 were examined in the Office Action under reply and stand rejected under (1) 35 U.S.C. §112, first paragraph (claims 1, 3, 5-7, 11-14 and 19-21); and (2) 35 U.S.C. §112, second paragraph (claims 19 and 20). These rejections are traversed and believed to be overcome for reasons discussed below.

Overview of the Above Amendments

Claims 19-21 have been amended to recite the invention with greater particularity. Specifically, the concluding statement in claims 19 and 20 has been amended to recite that the method treats or prevents "cerebellar neuronal degeneration." Additionally, claims 19 and 21 have been amended to recite that administration is to "a cerebellar lobe" of the subject. Support for these amendments can be found in the claims as filed, as well as throughout the specification at e.g., page 25, line 13.

Rejections Under 35 U.S.C. §112, First Paragraph

The Office rejected all pending claims under 35 U.S.C. §112, first paragraph as nonenabled. With respect to claims 1-3, 5, 11 and 12, the Office correctly notes these claims encompass both *in vitro* and *in vivo* applications but argues the "only *in vivo* use asserted is for gene therapy" and "the specification fails to adequately teach how to use the claimed methods therapeutically." Office Action, page 3. The Examiner further states: "The specification must teach how to use the claimed invention *in vivo*." Office Action, page 5. Regarding claims 6, 7, 13, 14 and 19-21, the Office argues these claims are exclusively directed to *in vivo* methods and do not encompass *in vitro* applications and are therefore not enabled. Office Action, page 7.

The Office supports this assertion citing Rubanyi et al., *Molec. Aspects Med.* (2001) 22:113-142 ("Rubanyi") as representing the state of the art at the time the application was filed. In particular, the Examiner asserts Rubanyi discusses the technical

hurdles that remain to be overcome in developing effective gene therapy techniques. These include problems with gene delivery vectors and improvement of gene expression control systems. Office Action, pages 4-5, bridging paragraph. However, applicants have overcome each of the hurdles described in Rubanyi. In particular, applicants have shown that *in vivo* administration using a lentiviral vector as the gene delivery system provides for efficient expression of the gene contained within the vector in Purkinje cells. As explained in the specification at page 33, lines 8-12:

> A two microliter injection ($10^4 - 10^5$ infectious units) into a single lobule transduced up to 1500 Purkinje cells. With an estimated 20,000 Purkinje cells in all 10 lobules of the mouse cerebellum approximately 10% of all Purkinje cells and close to 100% of the injected lobule were transduced.

The transduction of the Purkinje cells, which are cerebellar neurons, was detected based on expression and biological activity of the β-galactosidase protein.

Nevertheless, the Examiner ignores this finding and states that applicants have taught only β-galactosidase expression and that one of skill in the art would not expect β-galactosidase expression to have a therapeutic effect. Contrary to the Examiner's assertions, applicants are not asserting that β-galactosidase provides a therapeutic effect. Rather, β-galactosidase is used as a measure of transduction efficiency and is indeed indicative of therapeutic efficacy. In fact, β-galactosidase is routinely used and relied on by scientists in the discipline of gene therapy as predictive of whether a particular gene delivery system can provide a therapeutic benefit when used to deliver a therapeutic gene of interest.

To evidence that the β-galactosidase gene (lacZ) is routinely used to study transgene delivery and expression and is indeed predictive of a therapeutic benefit, applicants are providing a number of papers and abstracts related to delivery of the lacZ gene to the CNS. All of these papers and abstracts are from well-respected, peer-reviewed journals and are therefore probative evidence of the credibility of the lacZ system for predicting therapeutic utility. For example, Alisky et al., *NeuroReport* (2000)

11:2669-2673 relates to the delivery of the lacZ gene with FIV vectors, using the identical system described in the present patent application. The authors conclude at page 2673:

> FIV and AAV5 efficiently transduce Purkinje cells and other cortical neurons with the exception of granule cells and show promise in correction of cerebellar degeneration both hereditary and acquired.

In fact, subsequent experiments proved these statements true. Using the same FIV delivery system as described in Alisky and in the present application, the authors were able to deliver and express tripeptidyl peptidase I (TPP-I), the enzyme deficient in classical late-infantile neuronal ceroid lipofuscinosis (LINCL), in Purkinje cells. This, in and of itself, provides evidence of the therapeutic benefit obtained using the claimed system.

Stein and Davidson, *Meth. Enzymol.* (2002) 346:433-454, also comment on the Alisky study above (and hence on the experiments described in the application) as follows: "Thus, an FIV vector encoding a therapeutic molecule has potential clinical value." Stein and Davidson, page 448.

Similarly, Brooks et al., *Proc. Natl. Acad. Sci. USA* (2002) 99:6216-6221, delivered both the lacZ gene and β-glucoronidase gene to adult β-glucoronidase-deficient mice using the FIV-based vectors described and used by the present inventors. As explained in the paper, the β-galactosidase gene was first delivered in order to evaluate the feasibility of the FIV system to transduce and ultimately express β-galactosidase in the CNS (see, pages 6218-6219, bridging paragraph). When the β-glucoronidase gene was delivered using the same system, therapeutic benefits were achieved. Indeed, a Commentary in the same journal touted the results using the system as "exciting" and commented that the results "are likely to have general implications for the treatment of CNS disease in LSD." Sly and Vogler, *Proc. Natl. Acad. Sci. USA* (2002) 99:5760-5762, 5760. The Commentary goes so far as to say "this study will likely be viewed as a landmark that took us well beyond the blood-brain barrier." Accordingly, Brooks and Sly

clearly evidence the usefulness of the β-galactosidase system for predicting therapeutic efficacy.

The Examiner previously disregarded Brooks, arguing that it was post-filing art and that one of skill in the art "would not have had the benefit" of the Brooks teachings. Office Action, page 6. However, the FIV system and methods used in Brooks are the same as taught in the present application and it is seminal that journal articles that post-date the applicants' filing date can indeed be provided as evidence of enablement, provided the methods used are the same or analogous. Moreover, although Brooks expressed the β-glucoronidase gene at therapeutic levels in the striatum, cerebral cortex, or hippocampus, Brooks still provides credible evidence that gene delivery methods as described in the present application can provide a therapeutic benefit and that β-galactosidase is predictive of this benefit.

Further evidence of the predictive value of β-galactosidase is shown by a number of additional references. In the interest of brevity, applicants provide three abstracts herewith directed to delivery of β-galactosidase to the CNS using various gene delivery systems. Hagihara et al., *Gene Ther.* (2000) 7:759-763, abstract, delivered the lacZ gene using an HVJ-AVE delivery system. The lacZ gene was highly expressed in a number of cells, including Purkinje cells. Based on the findings using the lacZ gene, the authors state: "We conclude that the infusion of HVJ-AVE liposomes into the cerebrospinal fluid (CSF) space is applicable for widespread gene delivery into the CNS of large animals." Similarly, Agudo et al., *Hum. Gene Ther.* (2002) 13:665-774 delivered the lacZ gene using an HSV-1 amplicon vector. Expression of the lacZ gene within Purkinje cells was persistent and was maintained for at least 40 days. The authors conclude: "These results demonstrate that HSV-1 amplicon vectors can effect safe and stable transgene expression in Purkinje cells in vivo, raising the possibility of using these vectors for long-term gene therapy of human cerebellar disorders." Additionally, Kyrkanides et al., *Brain Res. Mol. Brain Res.* (2003)

119:1-9, successfully delivered and expressed the lacZ gene in Purkinje cells using an FIV vector.

Each of the above-described studies evidences the credibility of using a β-galactosidase system to predict the therapeutic benefit in a gene therapy context. It is well settled that art recognized screening procedures and tests can be relied on to establish utility under 35 U.S.C. §112, first paragraph. As explained in *In re Brana*, 34 USPQ 1436, 1442-1443, in addressing a 35 U.S.C. §112, first paragraph rejection:

> Usefulness in patent law, and in particular in the context of pharmaceutical inventions, necessarily includes the expectation of further research and development. The stage at which an invention in this field becomes useful is well before it is ready to be administered to humans. Were we to require Phase II testing in order to prove utility, the associated costs would prevent many companies from obtaining patent protection on promising new inventions, thereby eliminating an incentive to pursue, through research and development, potential cures in many crucial areas such as the treatment of cancer.

Likewise, rigorous correlation between disclosed *in vitro* utility and an *in vivo* activity is not necessary where the disclosure of pharmacological activity is reasonable based upon probative evidence. *Cross v. Iizuka*, 224 USPQ 739, 747 (Fed. Cir. 1985).

Not only have applicants provided probative evidence that the β-galactosidase system is considered predictive of therapeutic efficacy, they have also provided probative evidence that using the identical system as described in the application, a therapeutic protein, TPP-I, was expressed in Purkinje cells and provided a therapeutic benefit (see, Alisky described above). Thus, contrary to the Examiner's assertions, applicants have indeed taught how to make and use the invention and have therefore satisfied the enablement requirement of 35 U.S.C. §112, first paragraph. Accordingly, this basis for rejection has been overcome and withdrawal thereof is respectfully requested.

Rejection Under 35 U.S.C. '112, First Paragraph

Claims 19 and 20 stand rejected under 35 U.S.C. §112, second paragraph as indefinite because "the conclusory statement is broader in scope than the scope of the preamble." Office Action, page 7. Claims 19 and 20 have been amended in order to make the concluding statement consistent with the preamble and now recite that the method is for treating or preventing "cerebellar neuronal degeneration." Thus, this basis for rejection has been overcome and withdrawal thereof is respectfully requested.

CONCLUSION

Applicants respectfully submit that the claims define an invention that is patentable over the art. Accordingly, a Notice of Allowance is believed in order and is respectfully requested. If the Examiner notes any further matters which she believes may be resolved by a telephone interview, she is encouraged to contact the undersigned attorney at (650) 493-3400.

Respectfully submitted,

Date: 6/14/04

By: _____
Roberta L. Robins
Registration No. 33,208
Attorney for Applicants

ROBINS & PASTERNAK LLP
1731 Embarcadero Road, Suite 230
Palo Alto, CA 94303
Telephone: (650) 493-3400
Facsimile: (650) 493-3440

Transduction of murine cerebellar neurons with recombinant FIV and AAV5 vectors

Joseph M. Alisky,[1] Stephanie M. Hughes,[1] Sybille L. Sauter,[3] Douglas Jolly,[3] Thomas W. Dubensky Jr,[3] Patrick D. Staber,[1] John A. Chiorini[4] and Beverly L. Davidson,[1,2,CA]

Program in Gene Therapy, Departments of [1]Internal Medicine and [2]Neurology, University of Iowa College of Medicine, Iowa City, IA 52242; [3]Chiron Corporation, Center for Gene Therapy, San Diego, CA 92121-1204; [4]Gene Therapy and Therapeutics Branch, NIDCR, Bethesda, MD 20892, USA

[CA,1]Corresponding Author and Address

Received 23 May 2000; accepted 7 June 2000

Our data demonstrate that vectors derived from recombinant feline immunodeficiency virus (rFIV) and adeno-associated virus type 5 (rAAV5) transduce cerebellar cells following direct injection into the cerebellar lobules of mice. Both recombinant viruses mediated gene transfer predominantly to neurons, with up to 2500 and 1500 Purkinje cells transduced for rAAV5 or rFIV-based vectors, respectively. The vectors also transduced stellate, basket and Golgi neurons, with occasional transduction of granule cells and deep cerebellar nuclei. rAAV5 also spread outside the cerebellum to the inferior colliculus and ventricular epithelium, while rFIV demonstrated the ability to undergo retrograde transport to the physically close lateral vestibular nuclei. Thus, AAV5 and FIV-based vectors show promise for targeting neurons affected in the hereditary spinocerebellar ataxias. These vectors could be important tools for unraveling the pathophysiology of these disorders, or in testing factors which may promote neuronal survival. *NeuroReport* 11:2669–2673 © 2000 Lippincott Williams & Wilkins.

Key words: Axonal transport; Gene therapy; Purkinje cells

INTRODUCTION

Degenerative diseases of the cerebellum, such as the autosomal dominant spinocerebellar ataxias (SCA), are potentially amenable to gene therapy if sufficient numbers of the appropriate neuronal populations could be transduced. The SCAs can result from loss of Purkinje cells, inferior olivary and pontine neurons and, to a lesser extent, granule cells [1,2]. Onset is typically from the fifth to seventh decade of life, with degeneration occurring over a decade. The underlying genetic defect in several types of the SCA (SCA-1, SCA-2, SCA-3, and SCA-7) causes polyglutamine tract expansion and a toxic gain of function in the encoded protein [3,4]. Genes encoding neuroprotective sequences could in theory prevent or slow the degenerative process in these disorders.

Previous investigations of gene transfer in the cerebellum have been limited to replication-deficient adenovirus vectors (rAd). rAd expressing β-galactosidase injected into the cerebellar cortex of mice transduced precerebellar neurons in the brain stem [5,6]. This occurred via retrograde axonal transport of virons from mossy fiber terminals in the cortex back to neuronal soma. However, within the cortex itself mainly glia were transduced, with minimal transduction of Purkinje cells or other classes of neurons. Viral vectors that transduce cerebellar neurons directly would be preferable for use in the study of the spinal cerebellar ataxias, and for testing therapies in representative animal models [7,8].

Replication incompetent recombinant lentiviral vectors derived from human immunodeficiency virus (rHIV) or feline immunodeficiency virus (rFIV) show tropism for neurons *in vitro* [9] and *in vivo* when injected into the cerebrum [10]. The recombinant lentivirus vectors remain capable of infecting non-dividing cells when deleted of accessory proteins [11,12]. In recent studies, rHIV vectors pseudotyped with the vesicular stomatitis glycoprotein G (VSV-g) envelope protein mediated gene transfer to a large number of striatal neurons when injected into non-human primate brain, with no apparent decline in transgene expression throughout the three month study [13]. Similar results have been found with FIV-based lentivirus vectors [14].

Recombinant adeno-associated viruses (rAAV) have also been shown to mediate gene transfer to neurons when injected into the rodent cerebrum [15–19]. AAVs are DNA dependoviruses, requiring helper viruses for productive infections [20]. Six different AAV serotypes have been isolated (AAV1–AAV6), but only three have been tested for their transduction properties in mammalian brain: rAAV2, rAAV4, and rAAV5.

Earlier studies showed that vectors derived from AAV2 efficiently transduce neurons immediate to the site of administration [16,19]. More recently, we demonstrated that rAAV5-based vectors are capable of diffusion within the mouse striatum well beyond the injection site [19]. Similar to rAAV2 vectors, rAAV5 predominantly trans-

duced neurons in the hippocampus, cortex, striatum and medial septum. In this study we asked whether rAAV5 and rFIV could similarly transduce neurons in the cerebellum.

MATERIALS AND METHODS

Preparation of viral vectors: Both the rFIV and rAAV5 vectors have been described previously [11,19]. Briefly, the rFIV contained mutant vif and orf2 sequences inhibiting their expression, and expressed *Escherichia coli* β-galactosidase (cytoplasmic expression) driven off a cytomegalovirus promoter (rFIVβgal). The rAAV5 contained native AAV5 ITRs and was packaged using a three-plasmid transfection system, including one encoding AAV5 rep and cap [19,21]. The rAAV5 expresses nuclear targeted *E. coli* β-galactosidase from a Rous sarcoma virus promoter (rAAV5βgal). rFIVβgal and rAAV5βgal titers were $\sim 1 \times 10^8$ and 5×10^9 i.u./ml, respectively. In some experiments, a neuronal tracer, cholera toxin subunit b (CTb, List Laboratories, CA), was added at 1 μg/μl to the viral suspension. CTb is the nontoxic subunit of cholera toxin and was previously used to define the limits of an injection site in experiments with pseudorabies virus [22]. In this study, CTb immunoreactivity allowed independent visualization of cerebellar injection sites. In this manner, transport and spread of recombinant virus outside of the injection site could be distinguished from transduction within the primary injection site.

Cerebellar injections and tissue preparation: All animal procedures were approved by the University of Iowa Animal Care and Use Committee. Young adult C57BL/6 mice weighing 20–25 g were anesthetized with ketamine/xylazine. A burr hole was drilled at the midline posterior occipital bone overlying the cerebellar anterior lobe. Pressure injections (2 μl total) were made into a single cerebellar lobule using a Hamilton syringe cemented with a glass micropipette tip. A total of 16 animals were injected with rFIVβgal; eight with rFIVβgal alone, and eight with rFIVβgal plus CTb. Eight animals were injected with rAAV5βgal (four rAAV5βgal and four AAV5βgal plus CTb). Animals were sacrificed at 3–6 (rFIVβgal) or 7 (rAAV5βgal) weeks after gene transfer and cerebella, brain stems and thoracolumbar spinal cords removed. Tissues were postfixed in 4% paraformaldehyde overnight at 4°C, cryoprotected for 1–3 days in 30% sucrose in phosphate buffered saline at 4°C and then sectioned on a cryostat at 50 μm (cerebellum/brain stem sagitally and spinal cord longitudinally).

Histochemistry and immunofluorescence: Every other section was processed for β-galactosidase activity using 5-bromo-4-chloro-4-indolyl β-D-galactoside (X-Gal) according to Davidson [19,23]. Transport and spread of virus was determined by comparing the X-gal processed sections to adjacent sections that had been processed for CTb immunohistochemistry. CTb immunochemistry was performed according to Alisky and Tolbert [24]. Briefly, sections were blocked overnight in 2% rabbit serum in Tris-buffered saline, followed by 24 h in goat anti-choleragenoid (List Laboratories, Campbell, CA) diluted 1:10 000. Sections were then incubated with biotinylated, rabbit anti-goat secondary antibodies and processed using an avidin–biotin–peroxidase substrate. Neuronal versus glial transduction was determined by dual immunofluoresence for β-galactosidase (BioDesign International, Kennebunk, ME) and neuronal (calbindin) or glial (glial fibrillary acidic protein; GFAP) markers [19] on free-floating sections. Both anti-calbindin and anti-GFAP were purchased from Sigma Biochemicals (St. Louis, MO), and were used at a concentration of 1:3000 or 1:2000, respectively. Immunofluorescence was evaluated using a Zeiss LSM 510 confocal microscope and associated software.

Cell counts: β-Galactosidase-expressing Purkinje cells were counted in every other 50 μm cerebellar section under a ×10 brightfield objective. Purkinje cells were selected for quantification because they can be quickly counted in thicker sections without stereological correction [25].

RESULTS

Cerebellar injections are complicated by the fact that stereotaxic coordinates cannot be used to precisely target the site of an injection; there is animal to animal variation in the size of cerebellar lobules, as well as their 3-dimensional orientation [26]. We therefore took advantage of CTb, a reagent commonly used to track neurons from their terminals or projections to their somata. CTb allowed us two advantages: first, it enabled us to determine the exact location of the injection. Second, it revealed the pool of potentially transducable neurons at an injection site. In mice injected with virus plus CTb, CTb immunoreactivity was found encompassing the targeted cerebellar lobule (Fig. 1a,b). In some cases injections encompassed the dorsal half of one lobule and the ventral half of another lobule,

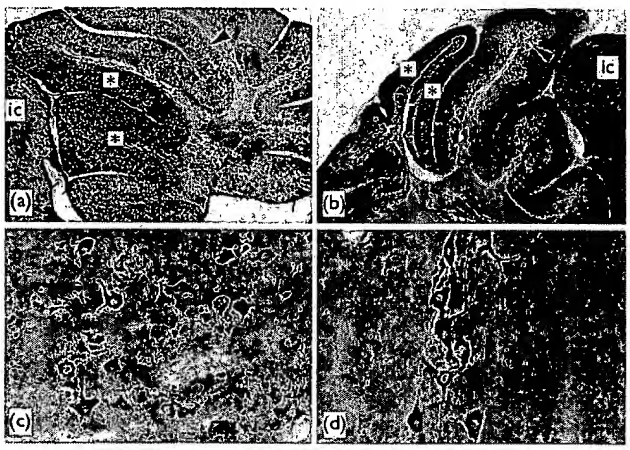

Fig. 1. Cholera toxin subunit b (CTb) injections into murine cerebella depict injection sites and retrogradely-labeled projection neurons. **(a)** Typical injection site visualized by CTb immunoreactivity. CTb is evident in the dorsal half of one lobule and the entire adjacent lobule (white asterisks). The inferior colliculus (ic) is labeled for orientation. **(b)** CTb-immunoreactivity in a section from a separate animal indicates a reduced area of exposure to CTb. The arrowheads in a and b point to mossy fiber terminals originating from cholera-labeled neurons in c and d. **(c)** Representative CTb immunoreactivity in precerebellar neurons (lateral reticular nuclei shown). **(d)** Retrogradely-labeled spinocerebellar neurons in the upper lumbar spinal cord.

while in other cases only a portion of a lobule was injected. At most, injections filled the molecular layer, Purkinje cell layer, granule cell layer and white matter of the arbor vitae but never extended to the deep cerebellar nuclei. Outside the injection site, the CTb retrogradely labeled precerebellar neurons in the cuneate, vestibular, olivary, reticular and spinal nuclei (Fig. 1c,d). Thus CTb co-injections mapped an extensive pool of neurons which could be potentially transduced via retrograde axonal transport of recombinant virus.

Recombinant FIVβgal was tested for its ability to transduce and undergo retrograde transport by cerebellar neurons. Histochemical assay for β-galactosidase activity in sections from cerebella harvested 3–6 weeks after injection of rFIVβgal into the cerebellar cortex indicated that rFIVβ gal mediated transduction to large numbers of neurons (Fig. 2a). The cytoplasmically targeted β-galactosidase was detected in the Purkinje cell somata, their dendritic arbors, and their axonal extensions (Fig. 2b,d) to the deep cerebellar and vestibular nuclei. The number of Purkinje cells transduced ranged from 78 to 1575 or 230 to 1298 in the rFIVβgal or rFIVβgal plus CTb-injected mice, respectively (Fig. 4). Thus, CTb had no profound effect on rFIV-mediated gene transfer. The number of transduced Purkinje cells was proportional to the area positive for CTb immunoreactivity. For example, in cerebella with fewer CTb-labeled neurons, there were fewer β-galactosidase-positive cells. Also, transduction was generally confined to the CTb-positive region. However, in some cerebella Purkinje cells in lobules adjacent to the injection site were also transduced.

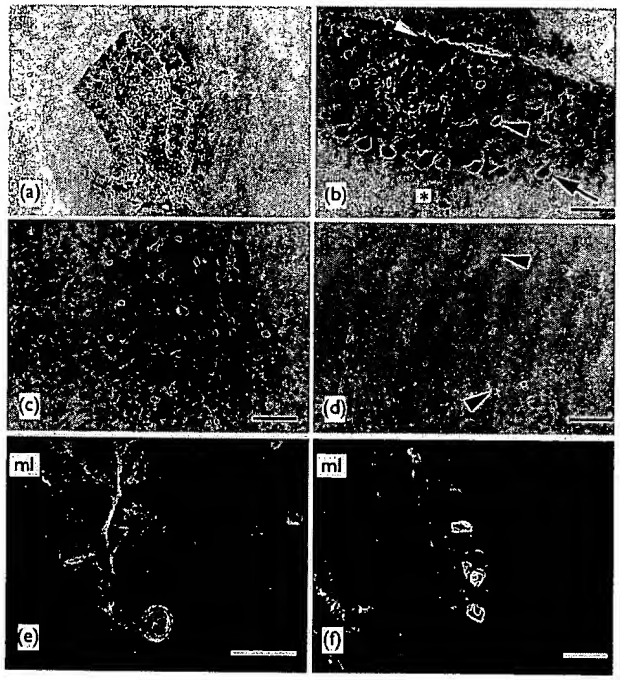

Fig. 2. rFIVβgal-mediated transduction of cells within mouse cerebellum. (**a**) Low power photomicrograph demonstrating transduction of neurons in all three cortical layers of mouse cerebellum. (**b**) Transduced row of Purkinje cells (arrow), with somata, dendritic trees and axons evident. One of the many β-galactosidase positive basket cell neurons (black arrowhead) and stellate neurons (white arrowhead) are depicted. Transduced golgi neurons within the granule cell layer are also shown (asterisk). (**c**) Transduced neurons in the deep cerebellar nuclei. (**d**) rFIVβgal mediated transduction of neurons (arrowheads) in the lateral vestibular nucleus by retrograde axonal transport of the virus from the cerebellar cortex. Purkinje cell axons are also seen. Bars in b–d = 50 μm. (**e**) Immunohistochemistry for β-galactosidase (green) and glial (red) markers show no overlap when evaluated by confocal microscopy. (**f**) Evaluation of confocal photomicrographs of sections immunostained for neuronal (red) and β-galacotosidase (green) markers demonstrate the neuronal tropism of rFIVβgal in the cerebellum. The molecular layer (ml) is noted. Bars in e and f = 30 μm.

In addition to Purkinje cells rFIVβgal transduced stellate and basket neurons in the molecular layer (Fig. 2b). In the granule cell layer, large numbers of fusiform Golgi neurons were transduced, but only scattered granule cell neurons expressed β-galactosidase (Fig. 2b). Retrograde transport of rFIVβgal was limited to deep cerebellar nuclei and lateral vestibular nuclei (Fig. 2c,d), which are the nuclei physically closest to the injection sites. Neurons in the cuneate, reticular, olivary and lumbar spinal nuclei were positive for CTb in rFIVβgal, CTb co-injected animals (Fig. 1c,d). However, these neurons were never β-galactosidase positive, indicating that rFIV has a limited ability to be retrogradely transported. Confocal microscopy of sections immunostained for neuronal or glial markers confirmed that cortical injections of FIVβgal resulted in exclusive transduction of neurons in the cortex (Fig. 2e,f).

Similar to rFIVβgal, rAAV5βgal was administered to the cerebellar cortex with or without CTb to determine the potential field of target neurons at the injection site. Again, there was no dramatic difference between rAAV5βgal plus CTb and rAAV5βgal alone, although there was much animal-to-animal variability (Fig. 4). There was extensive rAAV5βgal-mediated gene transfer to Purkinje cells, stellate and basket cells and Golgi neurons, and only minimal β-galactosidase expression in granule cell neurons (Fig. 3). The number of Purkinje cells transduced ranged from 12 to 2469 for rAAV5βgal alone and 447 to 1301 for rAAV5βgal plus CTb (Fig. 4). Evaluation of sections processed using immunohistochemistry for Purkinje or glial markers and β-galactosidase revealed that transduction was neuronal (Fig. 3e,f).

Compared with rFIVβgal, rAAV5βgal showed less retrograde transport. The only retrograde transport was to deep cerebellar nuclei (Fig. 3c). However, rAAV5βgal demonstrated greater distribution than rFIVβgal (compare Fig. 2a and Fig. 3a). Also, expressing cells could be seen in the overlying inferior colliculi (Fig. 3d). This indicates physical spread rather than axonal transport, as there are no axonal projections from the inferior colliculus into the cerebellum. This is consistent with the extensive distribution of rAAV5βgal noted following intrastriatal injections [19].

Direct counting of β-galactosidase-positive Purkinje cells in cerebella injected with rFIVβgal or rAAV5βgal give similar results (Fig. 4). Of note, the titer of rAAV5βgal was ~1 log higher than the rFIVβgal virus. Nonetheless, $> 10^3$ Purkinje cells, and numerous other neurons (Fig. 2, Fig. 3) were transduced upon introduction of 10^5–10^6 i.u. rFIV or rAAV. These data indicate that both rFIV and rAAV5-based vectors efficiently transduce cerebellar neurons.

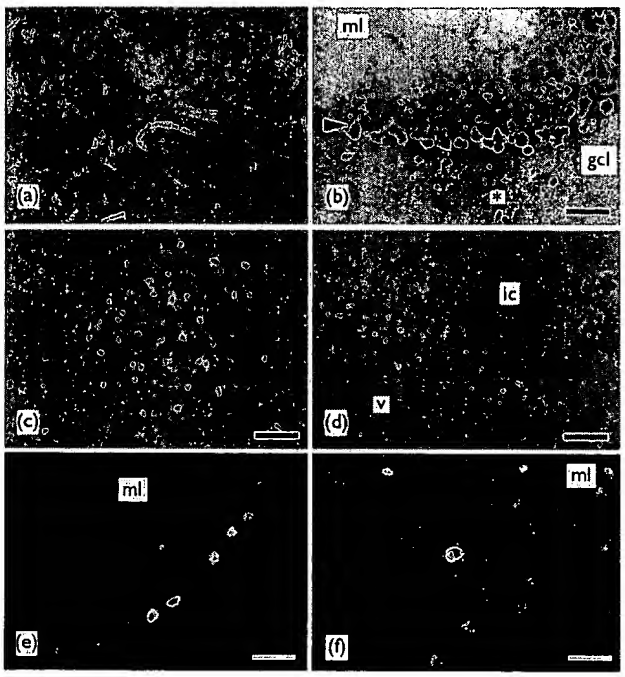

Fig. 3. rAAV5βgal-mediated gene transfer to cerebellar cells. (**a**) Low power photomicrograph demonstrating rAAV5βgal-mediated gene transfer to multiple lobules after a single injection. (**b**) Transduced nuclei of Purkinje (arrowhead) and the stellate and basket neurons in the molecular layer (ml) are easily discerned. rAAV5βgal also transduced a smaller number of Golgi neurons (asterisk) in the granule cell layer (gcl). (**c**) β-Galactosidase positive neurons in the deep cerebellar nuclei. (**d**) rAAV5βga spread outside the cerebellum to the inferior colliculi (ic). Bars in b–d = 50 μm. (**e**) Confocal photomicrographs following immunohistochemistry for β-galactosidase (green) and glial (red) markers show no overlap. (**f**) Confocal photomicrographs following immunohistochemistry for β-galactosidase (green) and Calbindin (red) markers demonstrate the Purkinje cell tropism of rAAAV5βgal in the cerebellum. Bars in e and f = 30 μm.

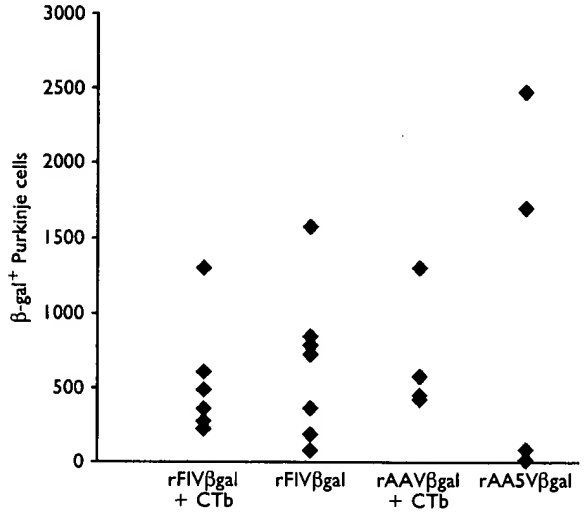

Fig. 4. Counts of transduced Purkinje cells. Each diamond represents an individual animal.

DISCUSSION

To our knowledge this is the first report of widespread direct gene transfer to cerebellar Purkinje cells. Previous reports with recombinant Ad showed limited Purkinje cell transduction mainly via retrograde transport of virus [5,6]. Our findings in the cerebellum were consistent with earlier results demonstrating transduction of cerebral neurons with recombinant lentivirus or AAV vectors [10,15–19,27]. In addition our data revealed selectivity among potential target neurons, a previously unknown characteristic of rFIV and rAAV5 vectors. This observation was probably made as a consequence of the morphologically distinct classes of neurons in each layer of the cerebellar cortex. Selective tropism could be more difficult to detect upon cerebral injection and gene transfer to the basal ganglia, because lamination and neuronal subtypes are more complex.

Within the molecular layer of a cerebellar lobule, stellate cells are located towards the outside while basket neurons are nearer the Purkinje cell bodies. The gigantic Purkinje cells form a monolayer, while fusiform Golgi neurons and small granule neurons are exclusively in the granule cell layer. Our data show that both rFIV- and rAAV5-based vectors transduce neurons in the molecular and Purkinje cell layer, with limited transduction of Golgi neurons and almost no gene transfer to granule neurons. As such we speculate that rAAV5 or rFIV vectors could be useful for studying or developing therapies for diseases in which Purkinje cells degenerate. On the other hand, these same vectors may be inappropriate for use in disorders resulting from loss of cerebellar granule cells.

The anterior lobe of the cerebellum receives input from multiple brain stem nuclei and all spinal cord segments. As such, injections into the cerebellum allow for direct evaluation of the ability of rAAV5- or rFIV-based vectors to undergo retrograde transport. We found that axonal transport with both rAAV5- and rFIV-based vectors was limited to the spatially closest nuclei. By contrast, adenovirus injected in the cerebellar cortex could be transported to the cuneate, lateral reticular and inferior olivary nuclei in the brain stem, with some limited transport to the lumbar spinal cord [5]. At this point, the inhibition of transport for rAAV5 or rFIV vectors relative to recombinant adenovirus remains unclear.

Further studies with thin sections (10–20 μm) and stereological sampling would be required for quantification of transduction of smaller neurons such as Golgi, stellate and basket cells and also to detect the small numbers of granule cells that are transduced. In this initial study, rAAV5βgal mediated transduction of the entire thickness of the molecular layer, but in the case of rFIVβgal, X-gal reaction product filled the Purkinje cell dendrites of the molecular layer, making visualization of stellate and basket cells difficult. However, our data provide an initial assessment of Purkinje cell transduction. A 2 microliter injection (10^5–10^6 i.u.) into a single lobule transduced up to 2500 Purkinje cells using rAAV5 vectors and 1500 cells using rFIV vectors. With an estimated 20 000 Purkinje cells in all 10 lobules of the mouse cerebellum [28] ~10% of all Purkinje cells and close to 100% of the injected lobule (Fig. 2, Fig. 3) were transduced. With injections into multiple lobules it is reasonable to assume that most or all Purkinje cells could

ultimately be transduced with rFIV- or rAAV5-based vectors. Thus, these vectors hold promise in discerning the underlying mechanisms of degeneration in diseases where Purkinje cells degenerate, such as the human disorders SCA-2 or SCA-6, or the murine cerebellar degeneration models [3,8,28,29].

CONCLUSION

FIV and AAV5 efficiently transduce Purkinje cells and other cortical neurons with the exception of granule cells, and show promise in correction of cerebellar degeneration both hereditary and acquired.

REFERENCES

1. Kato S, Hayashi H, Mikoshiba K *et al. Acta Neuropathol* **96**, 67–74 (1998).
2. Koeppen AH. *J Neuropathol Exp Neurol* **57**, 531–543 (1998).
3. Klockgether T and Evert B. *Trends Neurosci* **21**, 413–418 (1998).
4. Paulson HL. *Am J Hum Genet* **64**, 339–345 (1999).
5. Terashima T, Miwa A, Kanegal Y *et al. Anat Embryol* **196**, 363–382 (1997).
6. Hashimoto M, Aruga J, Hosoya Y *et al. Hum Gene Ther* **7**, 149–158 (1996).
7. Vig PJ, Subramony SH, Qin Z *et al. J Neurol Sci* **174**, 100–110 (2000).
8. Lorenzetti D, Watase K, Xu B *et al. Hum Mol Genet* **9**, 779–785 (2000).
9. Poeschla EM, Wong-Staal F and Looney DJ. *Nature Med* **4**, 354–357 (1998).
10. Naldini L, Blomer U, Gallay P *et al. Science* **272**, 263–267 (1996).
11. Johnston JC, Gasmi M, Lim LE *et al. J Virol* **73**, 4991–5000 (1999).
12. Zufferey R, Nagy D, Mandel RJ *et al. Nature Biotechnol* **15**, 871–875 (1997).
13. Kordower JH, Bloch J, Ma SY *et al. Exp Neurol* **160**, 1–16 (1999).
14. Davidson BL, Brooks A, Stein CS, *et al. Mol Ther* **1**, S 250 (2000).
15. Skorupa AF, Fisher KJ, Wilson JM *et al. Exp Neurol* **160**, 17–27 (1999).
16. Bartlett JS, Samulski RJ and McCown TJ. *Hum Gene Ther* **9**, 1181–1186 (1998).
17. Lo WD, Qu G, Sferra TJ *et al. Hum Gene Ther* **10**, 201–213 (1999).
18. Mandel RJ, Rendahl KG, Spratt SK *et al. J Neurosci* **18**, 4271–4284 (1998).
19. Davidson BL, Stein CS, Heth JA *et al. Proc Natl Acad Sci USA* **97**, 3428–3432 (2000).
20. Berns KI. Parvoviridae and their replication. In: Fields BN and Knipe DM, eds. *Virology*. New York: Raven Press Ltd, 1990: 1743–1763.
21. Chiorini JA, Wendtner CM, Urcelay E *et al. Hum Gene Ther* **6**, 1531–1541 (1995).
22. Chen S, Yang M, Miselis RR and Aston-Jones G. *Brain Res* **838**, 171–183 (1999).
23. Davidson BL, Doran SE, Shewach DS *et al. Exp Neurol* **125**, 258–267 (1994).
24. Alisky JM and Tolbert DL. *J Neurosci Methods* **52**, 143–148 (1994).
25. Tolbert DL, Ewald M, Gutting J and LaRegina MC. *J Comp Neurol* **335**, 490–507 (1995).
26. Larsell O. Albino rat. In: Jansen J, ed. *The Comparative Anatomy and Histology of the Cerebellum from Monotremes Through Apes*. Minneapolis: University of Minnesota Press, 1968; 31–58.
27. Blömer U, Naldini L, Kafri T *et al. J Virol* **71**, 6641–6649 (1997).
28. Caddy KWT and Biscoe TJ. *Brain Res* **111**, 396–398 (1976).
29. Heintz N and De Jager PL. *Ann NY Acad Sci* **868**, 502–514 (1999).

Acknowledgements: Research supported by the NIH (HD33531, NS34568), Iowa Center on Aging, the Roy J. Carver Foundation (BLD), the Amyotrophic Lateral Sclerosis Association (J.A.), and the University of Iowa Center on Aging (J.A., B.L.D.). Vectors produced at the U of I Gene Transfer Vector Core. Authors thank C.I. van de Wetering and T.A. Derksen for technical assistance.

Functional correction of established central nervous system deficits in an animal model of lysosomal storage disease with feline immunodeficiency virus-based vectors

Andrew I. Brooks*†‡, Colleen S. Stein§, Stephanie M. Hughes§, Jason Heth¶, Paul M. McCray, Jr.‖, Sybille L. Sauter**, Julie C. Johnston**, Deborah A. Cory-Slechta*†, Howard J. Federoff†‡††, and Beverly L. Davidson§†††‡§§

Departments of §Internal Medicine, ‡‡Neurology, §§Physiology and Biophysics, ¶Neurosurgery, and ‖Pediatrics, University of Iowa Program in Gene Therapy, University of Iowa College of Medicine, Iowa City, IA 52242; *Department of Environmental Medicine, †Centers for Functional Genomics, and ‡Aging and Developmental Biology, University of Rochester Medical Center, Rochester, NY 14642; and **Chiron Technologies, Center for Gene Therapy, San Diego, CA 92121

Edited by Roscoe O. Brady, National Institutes of Health, Bethesda, MD, and approved February 20, 2002 (received for review January 8, 2002)

Gene transfer vectors based on lentiviruses can transduce terminally differentiated cells in the brain; however, their ability to reverse established behavioral deficits in animal models of neurodegeneration has not previously been tested. When recombinant feline immunodeficiency virus (FIV)-based vectors expressing β-glucuronidase were unilaterally injected into the striatum of adult β-glucuronidase deficient [mucopolysaccharidosis type VII (MPS VII)] mice, an animal model of lysosomal storage disease, there was bihemispheric correction of the characteristic cellular pathology. Moreover, after the injection of FIV-based vectors expressing β-glucuronidase into brains of β-glucuronidase-deficient mice with established impairments in spatial learning and memory, there was dramatic recovery of behavioral function. Cognitive improvement resulting from expression of β-glucuronidase was associated with alteration in expression of genes associated with neuronal plasticity. These data suggest that enzyme replacement to the MPS VII central nervous system goes beyond restoration of β-glucuronidase activity in the lysosome, and imparts improvements in plasticity and spatial learning.

Mucopolysaccharidosis type VII (MPS VII), or Sly syndrome, is a lysosomal storage disease (LSD) resulting from mutations in β-glucuronidase (1). Similar to many other LSDs, there are both systemic and central nervous system (CNS) components, and patients show progressive disease involvement. In β-glucuronidase deficiency, lysosomes become progressively laden with undegraded glycosaminoglycans, leading to mental retardation and loss of hearing and vision. Patients also suffer from dysostosis multiplex, joint abnormalities, and hepatosplenomegaly.

An animal model for MPS VII, the β-glucuronidase-deficient gus^{mps} mouse (2, 3), has been invaluable in testing enzyme, cell, and gene therapies (4–9). MPS VII mice exhibit progressive lysosomal accumulation in multiple organs, including but not limited to bone, spleen, liver, lung, kidney, retina, and brain (2, 10). Early work by Birkenmeier and colleagues (11, 12) demonstrated that enzyme replacement therapy or bone marrow transplant was sufficient for correction of the visceral manifestations of the disorder. However, there remained significant storage within the brain. Until recently, the functional affect of CNS storage was unknown. Recent work by Sands and coworkers (13) demonstrated that MPS VII mice have progressive learning impairment as measured by a Morris water maze, as well as gradual loss of vision. Protection against the onset of storage pathology and the functional deficits of learning impairment could be accomplished by enzyme therapy or adeno-associated viral (AAV)-mediated therapy, initiated immediately after birth (13–15). Similarly, in an animal model of metachromatic leukodystrophy, HIV-based vectors could protect against disease incipience (16).

The collective incidence of LSDs is ≈1 in 7,000 live births, with 65% affecting the CNS (17). In most instances, disease diagnosis occurs well after the onset of pathology. As such, recovery of function, rather than protection from disease onset, will be an important benchmark for efficacy of gene therapy for the LSDs. Our prior studies with recombinant adenoviruses, and others' using AAV- or HIV-based vectors, established that focal expression of β-glucuronidase within diseased rodent striata could reduce storage pathology in both hemispheres (4, 5, 8, 18). The enzyme is secreted from transduced cells and taken up by nontransduced cells, leading to a zone of correction that extends beyond the site of transduction. In this study we set out to test the hypothesis that recombinant viral vectors based on feline immunodeficiency virus (FIV) could revert not only the pathologic phenotype, but more importantly, the established behavioral dysfunction.

Materials and Methods

***In Vivo* Delivery and Transgene Assays.** Animal studies were approved by the Animal Care and Use Committee of the University of Iowa. β-Glucuronidase-deficient mice were from The Jackson Laboratories and our own breeding colony. C57BL/6 mice were from Harlan Sprague (Indianapolis, IN). For virus injections, mice were anesthetized with ketamine/xylazine (100–125 mg/kg ketamine/10–12.5 mg/kg xylazine). The bregma was exposed by incision and used as a zero coordinate for stereotactic injections into the striatum or ventricle as described (4, 19).

For histological studies, the mice were injected unilaterally with 5 μl [1×10^6 transducing units (TU)] of vector. Animals were killed 3, 6, 9, 15, and 18 weeks later, and their brains were analyzed for enzyme activity, volume, *in situ* RNA hybridization, and storage vacuoles as described (4).

Vectors. FIV packaging constructs were generated from the full-length FIV molecular clone, FIV-34TF10 (National Institutes of Health AIDS Research and Reference Reagent Program), as described (20). The FIV construct, $pVET_LC\beta gal$ ($pVET_LC\beta$; ref. 20), was generated by inserting cytomegalovirus (CMV)-β-galactosidase into the $pVET_L$ FIV backbone. To construct

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: MPS VII, Mucopolysaccharidoses type VII; FIV, feline immunodeficiency virus; LSD, lysosomal storage disease; CNS, central nervous system; TU, transducing units; CMV, cytomegalovirus; RAPC, repeated acquisition and performance chamber; RA, repeated acquisition; P, performance; QPCR, quantitative real time PCR; MCS, multiple cloning site; RSV, Rous sarcoma virus.

See commentary on page 5760.

††To whom reprint requests may be addressed. E-mail: beverly-davidson@uiowa.edu or howard_federoff@urmc.rochester.edu.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

www.pnas.org/cgi/doi/10.1073/pnas.082011999

pVET$_L$RβgluC, a Rous sarcoma virus (RSV) promoter was isolated from pUC19RSV (J. K. Yee, personal communication), and the resulting fragment was inserted into pVET$_L$ to generate pVET$_L$RSV. The β-glucuronidase cDNA was isolated from pAdRSV4 (21) and ligated into pVET$_L$RSV to make pVET$_L$RβgluC(+polyA). The poly(A) tail was removed to generate pVET$_L$RβgluC. For FIVMCS, PVET$_L$RSV was modified to contain a multiple cloning site (MCS) downstream from the RSV promoter. The vesicular stomatitis virus (VSV)-G envelope plasmid, pCMV-G, has been described (22). Pseudotyped FIV vectors expressing β-glucuronidase (FIVβgluc) and β-galactosidase (FIVβgal) vector particles were done by transient transfection of plasmids into 293T cells plated one day earlier at 2.8×10^6 cells per 10-cm dish. Cotransfections were made with a 1:2:1 molar ratio of FIV packaging, FIV vector, and pCMV-G constructs. Harvested supernatants (32 and 48 h later) were passed through 0.45-μm Nalgene filters and concentrated by centrifugation (23). Vector titers determined by serial dilution on HT1080 cells and by quantitative PCR of transduced target cells ranged from 1×10^8 to 2×10^8 TU/ml (20).

Repeated Acquisition and Performance Chamber (RAPC) Analyses. The RAPC used to assess spatial learning and memory was done essentially as described (24–26). Mice ($n = 8$ each; MPS VII and control mice) were habituated to a reward solution before introduction to the RAPC. The habituation protocol required water deprivation for 12–16 h followed by exposure to a 0.2% saccharin solution for 30 min twice a day for 2 days, after which regular drinking water was provided *ad libitum*. Mice were introduced to the RAPC during four habituation sessions followed by four baseline experimental sessions (see Fig. 3, baseline sessions 1–4). A 12-h water deprivation period preceded behavioral sessions, with *ad libitum* water on nontest days. A session was 3 presentations each of the repeated acquisition (RA) component and the performance (P) component with 3 trials per presentation. In the RA component, the specific door sequence changed unpredictably with each successive session. During all P component sessions, the sequence of doors leading to the saccharin reward was identical. A discriminative stimulus (static audio signal) was played to signal that the P component was in effect. Thus, there were a total of 18 trials per session (3 trials per presentation × 3 presentations per component × 2 components per session). Latency was measured as the time required for the mouse to leave the start box, navigate through the four compartments, and consume the saccharin solution in the goal box. Mice were placed manually in the goal box if they failed to reach it within 10 min on any trial. Errors were defined as attempts to go through a locked door. Five weeks after the pretreatment sessions, MPS VII and control mice were split into groups ($n = 4$ per each treatment) and injected with FIVβgluc or FIVβgal (1×10^6 TU) into the striatum. Four weeks later, mice were reassessed according to the above protocol. Sessions 4, 5, and 6 were separated by 3 days each. A timeline for behavioral testing is detailed in Fig. 3*A*.

Statistics for Behavioral Analyses. Baseline differences (pretreatment) in errors and latencies were evaluated by using repeated-measures ANOVA (RMANOVA) with component (RA and P) and session (1–4) as within group factors and β-glucuronidase status (control vs. MPS VII) as a between-group factor. These were followed, where appropriate, by one-factor ANOVAs (β-glucuronidase status) for individual session data. Statistical assessment of changes in these measures after treatment were carried out separately for each treatment (FIVβgal and FIVβgluc) and for each component (RA and P) in RMANOVAs with β-glucuronidase status (control vs. MPS VII) as a between-group factor and session as within group factors. Subsequent one-factor ANOVAs were used, where appropriate, for determining differences between control vs. MPS VII groups for each session.

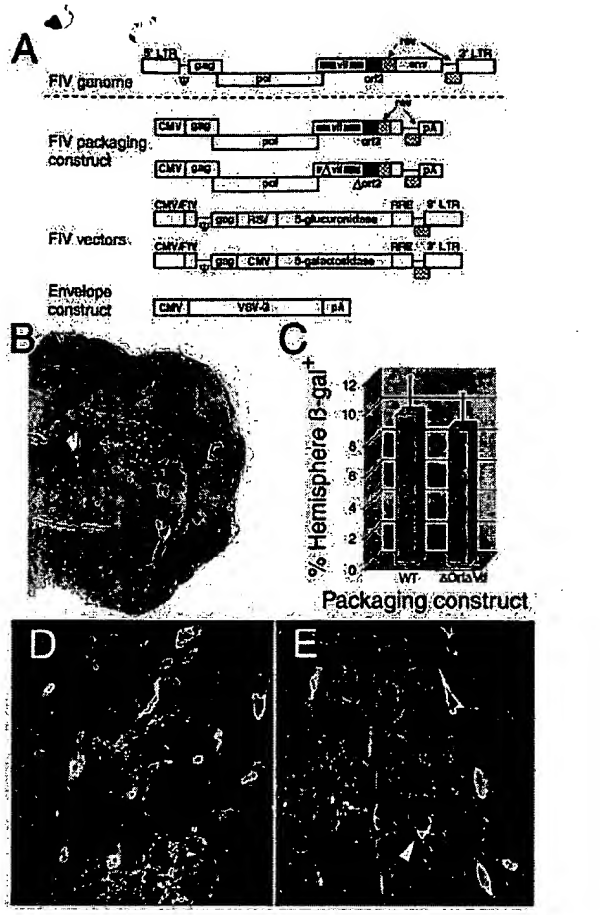

Fig. 1. FIV accessory proteins are not required for transduction of rodent CNS. FIV vectors encoding β-galactosidase containing both or neither of the Vif and Orf2 accessory proteins were generated as described (*Materials and Methods*) and injected into mice striata. (*A*) FIV packaging, vector, and envelope constructs. (*B*) Photomicrograph of a representative section stained for β-galactosidase activity. Mice were injected with FIVβgalΔvifΔorf2 18 weeks earlier. (*C*) The volume of β-galactosidase expression in FIV-injected hemispheres. (*D*) A representative confocal photomicrograph of the injected striatum after immunohistochemical staining for β-galactosidase (green) and NeuN (red) antigens. Cell soma colabeled for β-galactosidase and NeuN appear yellow in this merged image. (*E*) Occasional transduced glia could be identified in sections stained for glial fibrillary acid protein (GFAP, red) and β-galactosidase (green; arrowhead).

Microarray Study. Both striata of 16-week-old mice were injected with either 5 μl of FIVβgluc or FIVMCS (5×10^5 to 1×10^6 TU total; $n = 8$ per treatment). Eight weeks later the mice were killed, the brain was removed, meninges were dissected away, and brains were cut at the level of the dorsal hippocampus midsagitally. Hippocampi were removed, and before total RNA isolation, two groups of four hippocampi each were pooled, homogenized in Trizol (GIBCO/BRL) by using a Pro-200 homogenizer (PRO Scientific, Oxford, CT) and frozen on liquid nitrogen. RNA was prepped by using the standard Trizol protocol and assessed by gel electrophoresis and spectrophotometry. Target preparation was performed as directed (Affymetrix, Santa Clara, CA) with all components generated throughout the procedure (cDNA, full-length cRNA, and fragmented cRNA) analyzed by gel electrophoresis to assess size distribution.

Gene expression analysis was done by the Affymetrix Mu11K high-density oligonucleotide array at the University of Rochester Microarray Core Facility. Hybridization, staining, washing, and scanning were performed per the manufacturer's protocol. All

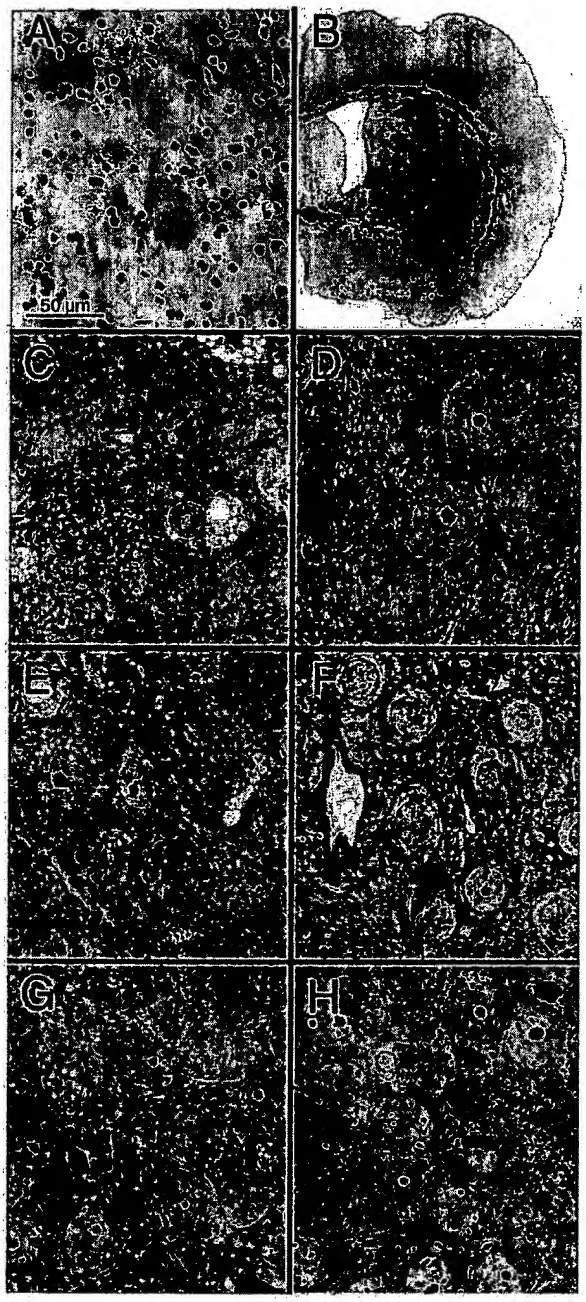

Fig. 2. β-Glucuronidase expression after FIV-mediated gene transfer. (*A*) Transgene-positive cells near the region of the injection as revealed by *in situ* RNA analyses. (*B*) β-Glucuronidase activity in the brain of a MPS VII mouse injected with FIVβgluc and stained for β-glucuronidase activity (dark red reaction product). (*C*, *E*, and *G*) Representative examples of the lysosomal storage in the striatum (*C*), cortex (*E*) and hippocampus (*G*) of 8- to 12-week-old MPS VII mice. (*D*, *F*, and *H*) Correction of the storage defect in the contralateral striatum (*D*), cortex (*F*) and hippocampus (*H*) 6 weeks after injection of FIVβgluc into an 8-week-old MPS VII mouse.

arrays were assessed for "array performance" by statistical analysis of control transcripts spiked into the hybridization mixture. In addition, several genes were identified on each array to assess the overall quality of array signal intensity. The results demonstrated that the arrays were within a 0.31-fold difference of each other at baseline, allowing for data normalization by the global scaling approach.

The Affymetrix Microarray Analysis Suite was used to generate the comparative analysis. The fold change represented for any transcript between the FIVMCS and FIVβgluc was calculated after global scaling to a target intensity of 2,500 (to normalize any differences in overall signal intensity among arrays). Super scoring was applied to all probe sets of 8 probe pairs or more. Data reported reflect the average fold changes in gene expression from two biological replicates (4 hippocampi each) for each condition. Data in Fig. 4*D* show the fold-change data from pair-wise comparisons of selected genes, of which all changed in the same direction. The false discovery rate of genes identified by our microarray analyses was determined by using significance analysis of microarrays (SAM) (27). A "two-class, unpaired" analysis within SAM was used to measure the effect of treatment as a function of genetically similar subjects (inbred strain, sex matched, littermates), and data were normalized by global scaling with $\Delta = 0.5$, based on the false positive distribution. By SAM, a total of 517 genes (4.7%) were called significant, and an additional 253 genes were called false positive. All genes discussed in Fig. 4 met SAM criteria for statistical significance.

Microarray Validation: Quantitative Real-Time PCR (QPCR) and Data Analysis. Levels of Pitpn (U96726), Fe65 (P46933), Fisp-12 (M70642), C/EBP (X61800), and Egr2 (Krox20, X06746) expression were examined in cDNAs archived from the microarray experiment by using TaqMan chemistry with specific probes and primers designed with PRIMER EXPRESS V.1.0. The following dye combinations were used: FAM (5-carboxyfluorescein; reporter), TAMRA (*N,N,N′,N′*-tetramethyl-6-carboxyrhodamine; quencher), and ROX (6-carboxy-X-rhodamine; reference). A validation experiment was done with a probe designed to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (M32599) to determine the relative probe efficiency. This probe was used as a reference gene for comparative analyses. The absolute value of the slope of log input amount vs. ΔC_T (C_T = threshold cycle) was less than 0.1 for all comparisons, allowing for $\Delta\Delta C_T$ determinations of relative quantitation of gene expression in FIVβgluc-treated mice vs. FIVMCS-treated mice (28). After optimization, cDNAs were diluted 1:100 with 1 μl used for each 25 μl PCR mixture containing 12.5 μl of ABI 2× Universal Master Mix, 1.25 μl of forward and reverse primers (final range 200–900, nM depending on primer set), and 1 μl of probe (final range 50–200 nM, depending on probe/primer set). Reactions were performed in triplicate and replicated three times. Thus, data in Table 1 reflect nine reactions per sample being tested; *t* test's for each sample achieved $P < 0.001$ or better. All reactions were run in an ABI 7700, and data were collected at all temperature phases during cycles. Raw data were analyzed by using the sequence detection software (Applied Biosystems) and relative quantitation using the comparative C_T method was performed in Microsoft EXCEL.

Results and Discussion

Evaluation of FIV Accessory Proteins for Gene Delivery to the CNS. FIV is distantly related to HIV. This nonprimate lentivirus does not replicate in human cells, and there has been no evidence of seroconversion among individuals exposed to FIV through repeated exposure by infected cats (29). FIV has two accessory proteins, Vif and Orf2. FIV Vif is the functional equivalent of HIV Vif, and is required for a productive infection in certain feline cells, for example, peripheral blood mononuclear cells (30, 31). FIV Orf2 is a weak transactivator of the FIV long terminal repeat (LTR), and therefore functionally similar to HIV Tat (32). We first determined whether Vif and Orf2 were required for gene transfer to mouse brain; vectors devoid of accessory proteins would be preferable if they are as functional as the parental vector *in vivo* (20). Because the FIV-based system uses a hybrid CMV/FIV LTR, we hypothesized that Orf2 would be dispensable, similar to that found *in vitro* (20). Recombinant FIV vectors expressing the reporter gene en-

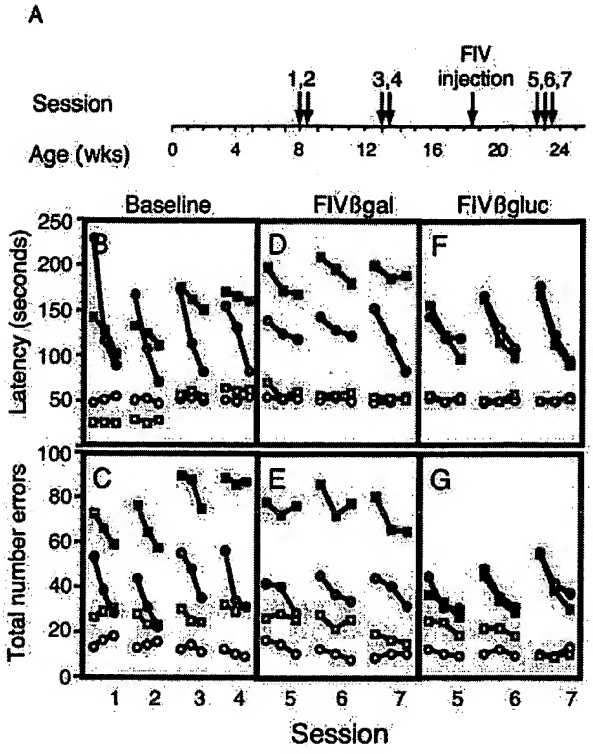

Fig. 3. Behavioral analyses in untreated and FIV-transduced mice. RAPC analysis was used to investigate baseline and posttreatment differences between MPS VII mice and age-matched heterozygous controls. (*A*) Behavioral sessions before and after gene transfer were performed according to the time line (*Materials and Methods*). MPS VII mice demonstrated significant baseline impairment in learning (solid red squares) relative to control mice (solid gray circles) as seen by both measures (latency in *B* and errors in *C*). There were no significant differences in the performance component of the assay between MPS VII (open red squares) and control (open gray circles) mice. After baseline testing, both groups were segregated randomly before bilateral striatal injection with FIVβgal or FIVβgluc. Behavioral tests on FIVβgal-treated (*D* and *E*) and FIVβgluc-treated (*F* and *G*) mice resumed 4 weeks after injection. FIVβgal-injected MPS VII mice continued to demonstrate a severe learning impairment in both latency and error (*D* and *E*). MPS VII mice injected with FIVβgluc exhibited no significant differences in learning compared with control mice in error or latency measures (*F* and *G*; $P \geq 0.05$), and reflect a recovery of the learning impairment seen in the baseline measurements (*B* and *C*). Solid symbols, learning component. Open symbols, performance component.

coding *Escherichia coli* β-galactosidase were generated by using the packaging constructs shown in Fig. 1*A* (20) and were injected into the striatum of mice. Full coronal sections were evaluated for β-galactosidase activity by histochemistry from 3 ($n = 6$; not shown) to 18 weeks ($n = 8$; Fig. 1*B*). In all cases, intrastriatal injections resulted in β-galactosidase activity in the ipsilateral striatum, corpus callosum, and the neocortex. Results with FIVβgalΔvifΔorf2 were not significantly different from FIVβgalwt ($P > 0.05$; Fig. 1*C*) as regards volume of transgene-positive brain (8.8 ± 2.4% and 9.7 ± 2.6% positive for FIVβgalΔvifΔorf2 and FIVβgalwt, respectively). Also, there was no decline in the β-galactosidase expression over time (8.8 ± 2.4% vs. 7.8 ± 1.7% positive at 3 and 18 weeks, respectively). Consistent with experiments with HIV-based vectors in rats (23), we found that the FIVβgal pseudotyped with the vesicular stomatitis virus (VSV)-G envelope readily transduced neuronal cells (Fig. 1*D*), with occasional glial fibrillary acidic protein, β-galactosidase double-positive (glial) cells also noted (Fig. 1*E*). Neuronal tropism was also found by using the amphotropic envelope from Moloney murine leukemia virus (data not shown).

FIV-Mediated Gene Transfer of β-Glucuronidase to MPS VII Mice. There are several important criteria for efficacy of FIV-mediated gene therapy to brain for treatment of the LSDs. First, does FIV-mediated gene transfer lead to adequate levels of β-glucuronidase expression for secretion and uptake into nearby nontransduced cells, and does the expressed enzyme lead ultimately to evidence of enzyme activity at sites distant from the injection? Second, is expression sufficient for reduction of the notable lysosomal pathology in affected brain in transduced and nontransduced cells? Finally, do FIV vectors expressing β-glucuronidase have a favorable impact on the disease course when introduced into animals with evident disease pathology and functional behavioral deficits? We first tested whether unilateral injection to the brains of 8-week-old MPS VII mice with the Vif- and Orf2-deleted FIVβgluc (Fig. 1*A*; $n = 6$) resulted in transduction of cells near the injection site. Although *in situ* hybridization for vector-encoded β-glucuronidase showed focal transduction (Fig. 2*A*), histological assay for β-glucuronidase (4) showed that levels of activity extended well beyond the area of transduced cells (Fig. 2*B*), with 18.1% ± 2.5% of the hemisphere positive for enzyme activity 18 weeks after vector introduction. In tissues from mice killed 3 weeks after gene transfer, histological correction of storage pathology was observed in the ipsilateral striatum and ipsilateral cortex, and modest reductions in storage product were seen in the contralateral cortex (data not shown). Six weeks after injection of FIVβgluc, there was notable correction of cellular morphology in regions of the injected (not shown) and contralateral hemispheres of the brain (Fig. 2 *D*, *F*, and *H*), as compared with the lysosome-laden cortical (Fig. 2*C*), striatal (Fig. 2*E*), or hippocampal (Fig. 2*G*) tissues from control MPS VII mice. The absence of lysosomal inclusions was maintained through the course of the study (18 weeks), supporting the hypothesis that persistent expression of β-glucuronidase from FIV-transduced cells, ≈2–5% of which may be secreted (13), resulted in correction of cells at increasing distances over time.

β-Glucuronidase-deficient mice have storage pathology in the eye and brain by several weeks after birth, leading to progressive decline in retinal and neuronal function as measured by electroretinograms or the Morris water maze, respectively (13, 33). By 8 weeks of age, there is sufficient motor impairment precluding further use of the Morris water maze. Thus, to test for functional recovery resulting from introduction of FIVβgluc into diseased mouse brain, the RAPC was used (24–26). The RAPC is a hippocampus-dependent paradigm that is capable of differentiating between spatial learning and other effects such as motor, motivational, and sensory disturbances that can confound analyses of spatial learning. Importantly, this testing paradigm allows for same-animal comparisons of pretreatment vs. posttreatment behavior in an environment devoid of strenuous physical activity.

Baseline testing. Behavioral testing using the RAPC was initiated on β-glucuronidase-deficient and age-matched heterozygous control littermates at 8 weeks of age, a time at which brain pathology is evident, to define baseline learning and performance abilities (Fig. 3*A*; *Materials and Methods*). Both latency (the time to reach the goal) and total errors (the number of mistakes made) were measured. In sessions 1 and 2, performed at 8 and 8.5 weeks of age, respectively, both MPS VII and control mice ($n = 8$ per group) showed reductions in the time to reach the goal box from the first to the last trial of each session (Fig. 3*B*). Similar results were seen for numbers of errors (Fig. 3*C*). However, the MPS VII mice showed significant impairments in learning relative to control mice (Fig. 3 *B* and *C*; $P = 0.0001$, $P = 0.029$ for latency and errors, respectively). At 13 and 13.5 weeks of age, β-glucuronidase-deficient mice showed further impairments in behavior, with increased latency and numbers of errors (sessions 3 and 4; Fig. 3 *B* and *C*). There was no evidence of impaired behavior of the control mice. Additionally, the relative difference in the performance component as compared with the learning component for either group remained unchanged ($P > 0.05$).

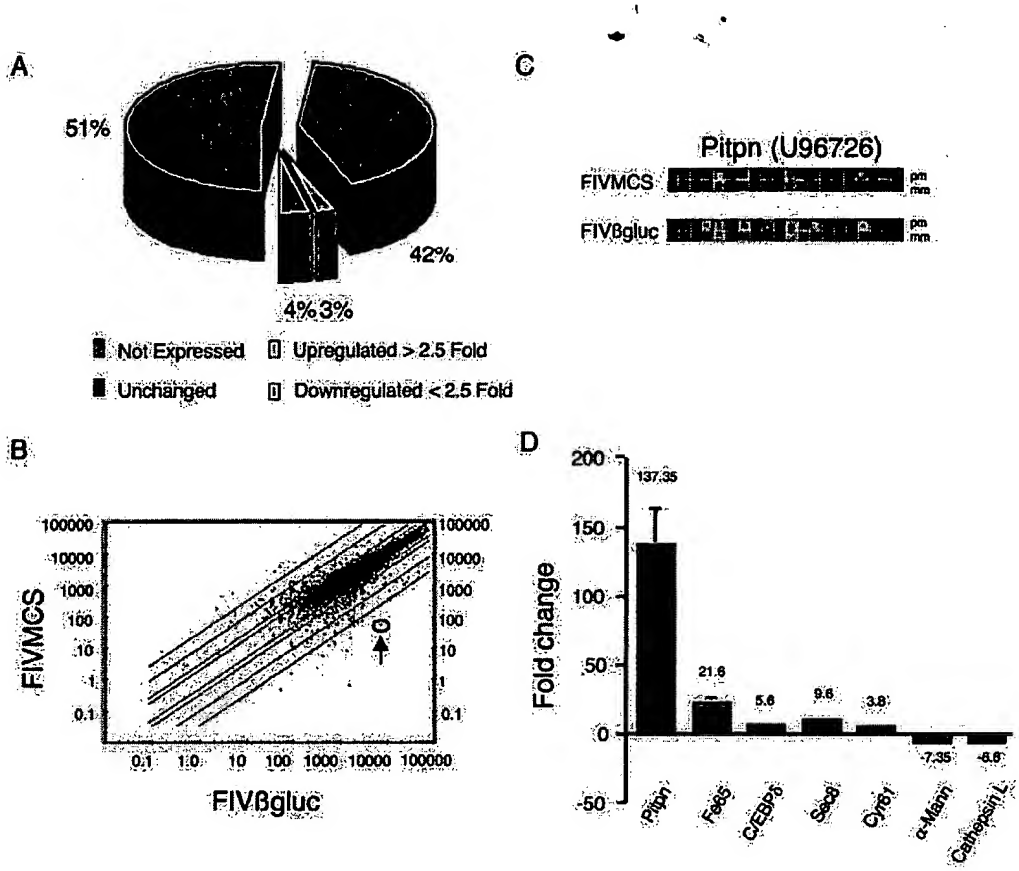

Fig. 4. Microarray analysis of murine hippocampus after treatment with FIV vectors. Hippocampi from FIVMCS- and FIVβgluc-treated animals were extracted 8 weeks after injection, and gene expression was analyzed on Affymetrix high-density oligonucleotide arrays. (*A*) A representation of the effects of FIVβgluc gene transfer on gene expression in the hippocampus. (*B*) A scatter plot depicts the average difference distribution of all genes examined, comparing FIVMCS with FIVβgluc samples. Red points depict genes that are called present, whereas blue points represent genes changing from absent to present or *vice versa*. Green lines indicate the magnitude of change with intervals of 2, 10, and 30-fold relative to baseline. One gene of interest, *Pitpn* (U96726), is circled in orange (arrow) and exhibits a significant change in gene expression as depicted by differences in the raw data images (*C*). (*C*) The increase in selective binding to the perfect match (pm) vs. the mismatch (mm) probes. (*D*) The increase or decrease in fold expression of RNA specific to selected genes from FIVβgluc relative to FIVMCS-treated mice. Data normalization and analyses were completed by using algorithms in the Affymetrix Microrarray Analysis suite and Data Mining Tools and significance analysis of microarrays (27).

Behavioral assays after FIV-mediated gene transfer. MPS VII and control mice were injected bilaterally at 18.5 weeks of age ($n = 4$ per group) with FIVβgluc or FIVβgal after baseline testing, with behavioral reassessment initiated 4 weeks later. β-Glucuronidase-deficient mice transduced with FIVβgluc showed dramatic improvements in latencies and errors to levels indistinguishable from controls (Fig. 3 *F* and *G*; $P > 0.05$). By contrast, FIVβgal-injected MPS VII mice continued to demonstrate severe learning impairments in error and latency measures relative to control mice (Fig. 3 *D* and *E*; $P = 0.0003$ and $P = 0.0019$ for error and latency, respectively). The data in Fig. 3 *F* and *G*, in concert with those shown in Fig. 2, suggest that therapies leading to reduction of storage pathology can significantly improve established learning deficits.

Molecular Correlates for Improved Pathology and Behavior. We next asked whether alterations in gene expression consistent with improvements in learning and memory or improvements in lysosomal function occurred as a result of FIVβgluc gene transfer. MPS VII mice (16 weeks of age) received intrastriatal injections of FIVβgluc or FIVMCS, a vector that expresses no transgene ($n = 8$ per group). FIVMCS, rather than FIVβgal, was used to avoid potential confounding effects caused by β-galactosidase expression. Eight weeks later, mice were killed, and RNA was isolated from dissected hippocampi. mRNAs were analyzed by using Affymetrix high-density oligonucleotide arrays.

After FIVβgluc injection into striatum, 93% of the genes and expressed sequence tags represented on the array did not change significantly relative to empty vector, or were not expressed, whereas 3% and 4% were up- or down-regulated greater than 2.5-fold, respectively (Fig. 4*A*). A scatter plot of average difference values between treatment groups illustrates the relative distribution of gene expression for the data set (Fig. 4*B*). Raw image data for *pitpn*, which encodes the α isoform of phosphatidylinositol transfer protein (PITPα), are shown in Fig. 4*C*. Reductions in *pitpn* expression occur in the mouse degeneration mutant *vibrator* (34), suggesting a requirement for PITPα in maintenance of neuronal function. In our studies, *pitpn* expression was increased approximately 137-fold relative to control tissues (Fig. 4*D*). QPCR validation studies confirmed elevated levels of mRNA specific to *pitpn* in isolated hippocampi (Table 1; refs. 28 and 35).

Although animals were killed 8 weeks after gene transfer, we found elevated expression of several genes implicated in learning and memory relative to FIVMCS-injected controls (Fig. 4*D*, Table 1). C/EBPδ has been shown to increase as a result of stimulation of cAMP signaling in hippocampal neurons (36, 37). In FIVβgluc-transduced mice, the expression of C/EPBδ was elevated 5.6- and 20-fold by microarray and QPCR analysis, respectively. *Egr2* (Krox 20), another immediate early gene implicated in learning and

Table 1. Validation of microarray data by QPCR

Gene of interest (GOI)	Treatment	GOI average C_T	GAPDH average C_T	ΔC_T GOI − GAPDH	$\Delta\Delta C_T$ $\Delta C_T - \Delta C_T$ MCS	Fold change relative to MCS
Pitpn (U96726)	FIVMCS	25.02 ± 0.80	19.18 ± 0.18	5.84 ± 0.82	0.00 ± 0.82	1.0
	FIVβgluc	20.07 ± 0.75	21.69 ± 0.46	−0.99 ± 0.89	−6.83 ± 0.88	114.04
Fe65 (P46933)	FIVMCS	28.62 ± 0.52	19.18 ± 0.18	9.44 ± 0.55	0.00 ± 0.55	1.0
	FIVβgluc	26.99 ± 0.77	21.69 ± 0.46	5.30 ± 0.90	−4.14 ± 0.90	17.67
C/EBPδ (X61800)	FIVMCS	31.71 ± 0.32	19.18 ± 0.18	12.53 ± 0.37	0.00 ± 0.37	1.0
	FIVβgluc	29.88 ± 0.87	21.69 ± 0.46	8.19 ± 0.99	−4.35 ± 0.99	20.30
Egr2 (X06746)	FIVMCS	22.35 ± 0.35	19.18 ± 0.18	3.17 ± 0.39	0.00 ± 0.39	1.0
	FIVβgluc	21.15 ± 0.51	21.69 ± 0.46	−0.54 ± 0.69	−3.71 ± 0.69	13.12

TaqMan chemistry was used to measure gene expression by means of real-time quantitative PCR (QPCR; *Materials and Methods*). A comparative C_T method allowed calculation of relative changes in gene expression of mice treated with FIVβgluc vs. FIVMCS. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference for all comparative analyses because microarray data revealed statistical consistency throughout experimental conditions and replicates. *t* tests on the replicates of each sample achieved $P < 0.001$ or better for every sample.

memory (38), was elevated approximately 10-fold by QPCR. *Egr2* is also important for peripheral nerve myelination (39). Expression of Cyr61, an extracellular matrix protein found in the human CNS, was shown to be induced on stimulation of m1 and m3 muscarinic acetylcholine receptors (40). We found a 3.8-fold increase in hippocampal Cyr61 mRNA.

Fe65 interacts with the γ-secretase-liberated tail of amyloid precursor protein (APP), and with the histone acetyltransferase Tip60 (41). Recent studies by Greengard and colleagues (42) showed that Fe65 also interacts with APP at the plasma membrane to foster axonal migration. In our studies, we noted significant elevations in Fe65 (Fig. 4*D* and Table 1). The role of Fe65 after FIVβgluc-mediated gene transfer is intriguing, and may imply enhanced neurite outgrowth and improved synaptic function. In support of this hypothesis, Sec8 expression was increased 9.6-fold (Fig. 4*D*). Sec8 is localized to regions of neurite outgrowth and is required for synaptogenesis (43).

Secondary elevations in lysosomal enzyme activity occurs in LSDs, and gene or enzyme therapy for the deficiency often normalizes these levels. For β-glucuronidase deficiency, enzyme replacement can reduce elevated α-galactosidase, β-galactosidase, and β-hexosaminidase levels (8, 11). It is currently unknown whether these secondary changes occur as a result of increased transcription or decreased protein degradation. We found that although reductions in RNA specific to β-hexosaminidase and α-galactosidase did not change greater than 2.5-fold, cathepsin L and α-mannosidase were significantly reduced (Fig. 4*D*). Whether this observation is reflective of the entire injected hemisphere, or only the harvested hippocampus, was not tested.

The combined results show that β-glucuronidase replacement reversed the severe neurological deficit in mice with established brain lysosomal storage disease. The data show that neuronal impairment has not occurred in aged MPS VII mice to a degree that function cannot be recovered. Before these studies, efficacy after gene, cell, or enzyme therapy to adult animal models with existing disease was assessed by clearance of the characteristic lysosomal distention observed within multiple cell types. We demonstrate that a functional assay of learning and memory is a more appropriate endpoint as we progress in the evaluation of vectors to treat human CNS disorders. Finally, our gene expression analyses using high-density oligonucleotide arrays and QPCR implied that β-glucuronidase treatment improved CNS function in manners beyond simple reconstitution of enzyme levels.

We thank Inês Martins, Qinwen Mao, Phil Sheridan, Kim Wahoski, and Christine McLennan for assistance, and Michael J. Welsh for critical discussions. This work was supported by National Institutes of Health Grants NS34568, DK54759, HD33531 (to B.L.D.), and MH57047 (to H.J.F.), and the Roy J. Carver Foundation (to B.L.D.).

1. Sly, W. S., Quinton, B. A., McAlister, W. H. & Rimoin, D. L. (1973) *J. Pediatr.* **82**, 249–257.
2. Birkenmeier, E. H., Davisson, M. T., Beamer, W. G., Ganschow, R. E., Vogler, C. A., Gwynn, B., Lyford, K. A., Maltais, L. M. & Wawrzyniak, C. J. (1989) *J. Clin. Invest.* **83**, 1258–1266.
3. Sands, M. S. & Birkenmeier, E. H. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 6567–6571.
4. Ghodsi, A., Stein, C., Derksen, T., Yang, G., Anderson, R. D. & Davidson, B. L. (1998) *Hum. Gene Ther.* **9**, 2331–2340.
5. Stein, C. S., Ghodsi, A., Derksen, T. & Davidson, B. L. (1999) *J. Virol.* **73**, 3424–3429.
6. Ghodsi, A., Stein, C., Derksen, T., Martins, I., Anderson, R. D. & Davidson, B. L. (1999) *Exp. Neurol.* **160**, 109–116.
7. Daly, T. M., Vogler, C., Levy, B., Haskins, M. E. & Sands, M. S. (1999) *Proc. Natl. Acad. Sci. USA* **96**, 2296–2300.
8. Bosch, A., Perret, E., Desmaris, N., Trono, D. & Heard, J. M. (2000) *Hum. Gene Ther.* **11**, 1139–1150.
9. Snyder, E. Y., Taylor, R. M. & Wolfe, J. H. (1995) *Nature (London)* **374**, 367–370.
10. Lazarus, H. S., Sly, W. S., Kyle, J. W. & Hageman, G. S. (1993) *Exp. Eye Res.* **56**, 531–541.
11. Vogler, C., Sands, M., Higgins, A., Levy, B., Grubb, J., Birkenmeier, E. H. & Sly, W. S. (1993) *Pediatr. Res.* **34**, 837–840.
12. Birkenmeier, E. H., Barker, J. E., Vogler, C. A., Kyle, J. W., Sly, W. S., Gwynn, B., Levy, B. & Pegors, C. (1991) *Blood* **78**, 3081–3092.
13. O'Connor, L. H., Erway, L. C., Vogler, C. A., Sly, W. S., Nicholes, A., Grubb, J., Holmberg, S. W., Levy, B. & Sands, M. S. (1998) *J. Clin. Invest.* **101**, 1394–1400.
14. Sands, M. S., Vogler, C., Torrey, A., Levy, B., Gwynn, B., Grubb, J. & Sly, W. S. (1997) *J. Clin. Invest.* **99**, 1596–1605.
15. Frisella, W. A., O'Connor, L. H., Vogler, C. A., Roberts, M., Walkley, S., Levy, B., Daly, T. M. & Sands, M. S. (2001) *Mol. Ther.* **3**, 351–358.
16. Consiglio, A., Quattrini, A., Martino, S., Bensadoun, J. C., Dolcetta, D., Trojani, A., Benaglio, G., Marchesini, S., Cestari, V., Oliverio, A., *et al.* (2001) *Nat. Med.* **7**, 310–316.
17. Meikle, P. J., Hopwood, J. J., Clague, A. E. & Carey, W. F. (1999) *J. Am. Med. Assoc.* **281**, 249–254.
18. Skorupa, A. F., Fisher, K. J., Wilson, J. M., Parente, M. K. & Wolfe, J. H. (1999) *Exp. Neurol.* **160**, 17–27.
19. Franklin, K. B. J. & Paxinos, G. (1997) *The Mouse Brain in Stereotaxic Coordinates* (Academic, San Diego), pp. 1–190.
20. Johnston, J. C., Gasmi, M., Lim, L. E., Elder, J. H., Yee, J.-K., Jolly, D. J., Campbell, K. P., Davidson, B. L. & Sauter, S. L. (1999) *J. Virol.* **73**, 4991–5000.
21. Davidson, B. L., Doran, S. E., Shewach, D. S., Latta, J. M., Hartman, J. W. & Roessler, B. J. (1994) *Exp. Neurol.* **125**, 258–267.
22. Yee, J. K., Miyanohara, A., LaPorte, P., Bouic, K., Burns, J. C. & Friedman, T. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 9564–9568.
23. Blömer, U., Naldini, L., Kafri, T., Trono, D., Verma, I. M. & Gage, F. H. (1997) *J. Virol.* **71**, 6641–6649.
24. Brooks, A. I., Cory-Slechta, D. A., Murg, S. M. & Federoff, H. J. (2000) *Neurobiol. Learn. Mem.* **74**, 241–258.
25. Brooks, A. I., Cory-Slechta, D. A., Bowers, W. J., Murg, S. L. & Federoff, H. J. (2000) *Hum. Gene Ther.* **11**, 2341–2352.
26. Brooks, A. I., Cory-Slecta, D. A. & Federoff, H. J. (2000) *Proc. Natl. Acad. Sci. USA* **97**, 13378–13383.
27. Tusher, V. G., Tibshirani, R. & Chu, G. (2001) *Proc. Natl. Acad. Sci. USA* **98**, 5116–5121.
28. PE Applied Biosystems (1997) *User Bulletin #2. ABI Prism 7700 Sequence Detection System*, Technical Notes (Perkin–Elmer, St. Pete Beach, FL), pp. 1–35.
29. Yamamoto, J. K., Hansen, H., Ho, E. W., Morishita, T. Y., Okuda, T., Sawa, T. R., Nakamura, R. M. & Pedersen, N. C. (1989) *J. Am. Vet. Med. Assoc.* **194**, 213–220.
30. Shacklett, B. L. & Luciw, P. A. (1994) *Virology* **204**, 860–867.
31. Tomonaga, K., Norimine, J., Shin, Y. S., Fukasawa, M., Miyazawa, T., Adachi, A., Toyosaki, T., Kawaguchi, Y., Kai, C. & Mikami, T. (1992) *J. Virol.* **66**, 6181–6185.
32. de Parseval, A. & Elder, J. H. (1999) *J. Virol.* **73**, 608–617.
33. Bastedo, L., Sands, M. S., Lambert, D. T., Pisa, M. A., Birkenmeier, E. & Chang, P. L. (1944) *J. Clin. Invest.* **94**, 1180–1186.
34. Hamilton, B. A., Smith, D. J., Mueller, K. L., Kerrebrock, A. W., Bronson, R. T., van Berkel, V., Daly, M. J., Kruglyak, L., Reeve, M. P., Nembauser, J. L., *et al.* (1997) *Neuron* **18**, 711–722.
35. Bustin, S. A. (2000) *J. Mol. Endocrinol.* **25**, 169–193.
36. Taubenfeld, S. M., Wiig, K. A., Monti, B., Dolan, B., Pollonini, G. & Alberini, C. M. (2001) *J. Neurosci.* **21**, 84–91.
37. Yukawa, K., Tanaka, T., Tsuji, S. & Akira, S. (1998) *J. Biol. Chem.* **273**, 31345–31351.
38. Pearse, D., Mirza, A. & Leah, J. (2001) *Brain Res.* **894**, 193–208.
39. Nagarajan, R., Svaren, J., Le, N., Araki, T. & Watson, M. (2001) *Neuron* **30**, 355–368.
40. Albrecht, C., von der Kammer, H., Mayhaus, M., Klaudiny, J., Schweizer, M. & Nitsch, R. M. (2000) *J. Biol. Chem.* **275**, 28929–28936.
41. Cao, X. & Südhof, T. C. (2001) *Science* **293**, 115–120.
42. Sabo, S. L., Ikin, A. F., Buxbaum, J. D. & Greengard, P. (2001) *J. Cell Biol.* **153**, 1403–1414.
43. Hazuka, C. D., Foletti, D. L., Hsu, S. C., Kee, Y., Hopf, F. W. & Scheller, R. H. (1999) *J. Neurosci.* **19**, 1324–1334.

Commentary

Brain-directed gene therapy for lysosomal storage disease: Going well beyond the blood–brain barrier

William S. Sly*† and Carole Vogler‡

*Edward A. Doisy Department of Biochemistry and Molecular Biology, and ‡Department of Pathology, Saint Louis University School of Medicine, 1402 South Grand Boulevard, St. Louis, MO 63104

The lysosomal storage diseases (LSDs) are a heterogeneous group of disorders that affect 1/7,000 live-born infants, the majority of which develop central nervous system (CNS) disease. Brooks *et al.* (1) report exciting results from Davidson's group with brain-directed gene therapy for murine mucopolysaccharidosis (MPS) VII that are likely to have general implications for the treatment of CNS disease in LSD. Each LSD results from a deficiency of a single lysosomal enzyme important for degrading macromolecules that must be turned over in lysosomes. More than 40 LSDs have been described (2). Over the past two decades, dramatic progress has been made in understanding the biogenesis, structure, and function of lysosomes and the processes by which newly synthesized acid hydrolases are assembled, processed, and transported to lysosomes.

Understanding the receptors that target enzymes to lysosomes led to the development of successful enzyme replacement therapy.

Understanding the receptors that target enzymes to lysosomes, some of which are expressed on the cell surface, led to the development of successful enzyme replacement therapy for one of the LSDs, Gaucher Disease, a disorder of sphingolipid degradation (3). Gaucher Disease results from deficiency of glucocerebrosidase (β-glucosidase), the enzyme involved in the last step of sphingolipid degradation. Storage of glucocerebroside in macrophages produces tremendous enlargement of spleen and liver, disabling bone involvement and occasional pulmonary incapacity. The strategy for treatment involved purification of placental enzyme and later recombinant enzyme from Chinese hamster ovary cell secretions and modification of the native enzyme to expose mannose residues on oligosaccharides. This strategy targets the infused enzyme to the mannose receptors of fixed-tissue macrophages, precisely the cells affected by the storage; receptor-mediated endocytosis delivers enzyme to the lysosomes where the substrate is stored. Over 3,500 Gaucher Disease patients have been treated since the early 1990s, and the treatment is considered a clinical success (4). The major form of Gaucher Disease does not have CNS involvement. However, the less common neuropathic forms of Gaucher Disease will require a strategy for the enzyme to reach the CNS.

Another apparent success in the treatment of LSD is enzyme therapy for Fabry Disease, another sphingolipid disorder that does not produce lysosomal storage in the CNS (5). This LSD affects primarily vascular endothelial cells and results from a deficiency of α-galactosidase A, which leads to the pathological accumulation of globotriaosylceramide (GL3) and related glycosphingolipids in these cells. Kidney involvement leads to loss of renal function in the third or fourth decade of life. This disease does not affect brain directly, so enzyme access to brain is not required, but it does eventually lead to cerebral vascular insufficiency because of endothelial damage. Two clinical trials of enzyme produced by two different companies were reported recently (6, 7). Although both appear very promising, long-term data are not yet available. Both products have been approved for clinical use in Europe, and approval for both has been sought in the United States.

The MPS storage disorders are also moving up to the plate for enzyme replacement with clinical trials for MPS I (Hurler Disease, α-L-iduronidase deficiency) already reported (8), trials for MPS II (Hunter Disease, α-L-iduronidate sulfate deficiency) under way, and trials for MPS VI (Maroteaux–Lamy Disease, *N*-acetylgalactosamine-4-sulfatase deficiency) are just beginning. Although MPS VII (Sly Disease, β-glucuronidase deficiency) may be among the last of these disorders to be treated, it played an important role in the evolution of enzyme replacement therapy for the whole group of LSDs (9). Because it appears unlikely that infused lysosomal enzymes will cross the blood–brain barrier, there is not much optimism that i.v. administered enzyme alone will correct the CNS storage present in most of the MPS disorders.

Like the MPS, most of the other LSDs also have CNS involvement and therefore require a strategy for getting enzyme beyond the blood–brain barrier to achieve correction. A number of groups using a variety of viral vectors and enzyme-producing cells have achieved expression of enzyme in brain of animal models (10–17). Impressive degrees of clearing of local, and in some cases distant, storage have been demonstrated. These experiments raised hopes that arresting progression of CNS pathology was possible through brain-directed gene therapy. What few dared to hope was that this approach would not only prevent progression but also erase neurologic deficits. That is just what Davidson's group reports (1).

The authors (1) show that established CNS storage and the related functional deficits in MPS VII mice can be ameliorated by viral-mediated gene therapy. The lentivirus feline immunodeficiency viral vector they used transduced terminally differentiated cells in the brain and mediated β-glucuronidase (GUSB) gene transfer into CNS cells in adult MPS VII mice. This treatment resulted in secretion of GUSB from transduced cells and uptake by nontransduced cells, leading to reduction in preexisting established brain LSD. Correlating with the reduction in storage in the CNS, these adult mice with

See companion article on page 6216.

†To whom reprint requests should be addressed. E-mail: slyws@slu.edu.

www.pnas.org/cgi/doi/10.1073/pnas.102175599

established behavioral abnormalities related to the lysosomal storage had dramatic improvement in spatial learning and memory when GUSB was expressed. Finally, the correction of the pathology and cognitive improvement were accompanied by changes in expression of genes that have been associated with neuronal plasticity. These observations are particularly important because, as the authors point out, most patients with LSD are not diagnosed until they have established lysosomal storage lesions and functional defects. Recovery of function rather than protection from disease onset is a key goal for any effective therapy for human LSD, because most patients are diagnosed well after onset of CNS disease.

The model the authors (1) chose to study is murine MPS VII, the mouse model for human MPS VII or Sly Disease, which results from GUSB deficiency (18). MPS VII is one of the rarest of the human mucopolysaccharide storage disorders, each of which is produced by deficiency in one of the enzymes involved in the degradation of glycosaminoglycans (GAGs), formerly called mucopolysaccharides. Its importance in the evolution of our understanding of lysosomal enzyme targeting outweighs its clinical significance. When first discovered in the early 1970s, MPS VII had one unique feature among the MPS disorders: the deficient enzyme, GUSB, had been purified and characterized several years before the disease was identified. Addition of GUSB was shown to prevent and correct the accumulation of GAGs in fibroblasts from MPS VII patients (19, 20). Thus, MPS VII immediately attracted attention as a model to study enzyme replacement therapy. Studies of the cultured skin fibroblast model system led to the discovery that uptake of GUSB depends on cell surface receptors that recognize phosphate-containing sugar moieties (Man6-P) on the enzyme (21). The Man6-P residues are added to the GUSB and other acid hydrolases as a means of targeting intracellular enzymes

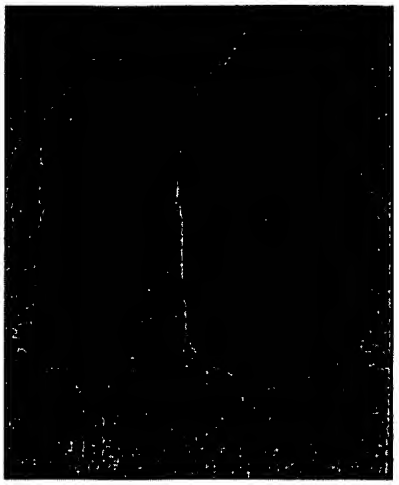

Fig. 1. An adult MPS VII (Sly Disease) mouse (*Left*) is much smaller than its phenotypically normal littermate (*Right*) and has facial dysmorphism with a broad shortened nose and short limbs.

to lysosomes. Another receptor was identified when injected GUSB, from which phosphate had been removed, was found to be rapidly taken up by fixed-tissue macrophage receptors that recognize exposed mannose residues (22). These studies paved the way for Brady and associates to develop "mannose-targeted" cerebrosidase for the treatment of Gaucher Disease (3, 4). These early findings naturally heightened hopes that enzyme replacement in MPS VII patients might lead to correction of lysosomal storage lesions in this disorder. However, MPS VII proved to be too rare (fewer than 100 cases recognized) and too variable to allow controlled experiments to evaluate the response to enzyme therapy.

Nonetheless, hopes for therapy for this and related disorders were greatly advanced by the discovery by Birkenmeier *et al.* at The Jackson Laboratory that GUSB deficiency in mice produces a disorder resembling Sly Disease in humans (23). (Fig. 1) MPS VII mice have a degenerative disease with progressive disability that reduces life span from an average of 28 to just 5 months. Progressive accumulation of undegraded glycosaminoglycans in lysosomes affects the spleen, liver, kidney, cornea, brain, heart valves, and skeletal system and produces widespread organ dysfunction. Progressive hearing loss leads to early deafness, and defects in learning and memory are evident (24–26).

The MPS VII mouse, with a well characterized and uniform genetic constitution and a relatively short life span, proved an attractive model to study experimental therapies for LSD. Mice with MPS VII responded well to bone marrow transplantation, although there was little reduction in brain storage vesicles (27, 28). Enzyme replacement using recombinant GUSB elicited dramatic improvements in visceral pathology but little change in the lyosomal storage lesions in brain unless given to newborns (29–32). Infused GUSB did not cross the blood–brain barrier in mice after 2 wk of age (32). Many promising studies have been reported recently by using this model to study gene therapy, including therapy for CNS storage (10–17). Brain-directed gene therapy, in which viral vectors were introduced directly into the brain, proved one way to bypass the blood–brain barrier, and several studies showed evidence of clearance of CNS storage. However, until now, none of these studies addressed the question raised by Brooks *et al.* (1): whether therapy that corrected the typical cellular pathology in brain could also erase preexisting neurological deficits. For this reason, the study by Davidson's group represents a major advance in this area.

Given the rapidly expanding number of animal models of LSD with CNS involvement and the generality of the biology of lysosomal enzyme transport, these studies are likely to be replicated in other animal models. If the results in other animal models turn out to be as promising as those presented for murine MPS VII, this study will likely be viewed as a landmark that took us well beyond the blood–brain barrier.

COMMENTARY

1. Brooks, A. I., Stein, C. S., Hughes, S. M., Heth, J., McCray, P. M., Jr., Sauter, S. L., Johnston, J. C., Cory-Slechta, D. A., Federoff, H. J. & Davidson, B. L. (2002) *Proc. Natl. Acad. Sci. USA* **99**, 6216–6221.
2. Scriver, C. R., Beaudet, A. L., Sly, W. S. & Valle, D., eds. (2001) *The Metabolic and Molecular Bases of Inherited Disease* (McGraw–Hill), 8th Ed., pp. 3371–3896.
3. Barton, N. W., Brady, R. O., Dambrosia, J. M., Di Bisceglie, A. M., Doppelt, S. H., Hill, S. C., Mankin, H. J., Murray, G. J., Parker, R. I. & Argoff, C. E. (1991) *N. Engl. J. Med.* **324**, 1464–1470.
4. Barranger, J. A. & O'Rourke, E. (2001) *J. Inherit. Metab. Dis.* **24** Suppl. 2, 89–96.
5. Desnick, R. J., Ioannou, Y. A. & Eng, C. M. (2001) in *The Metabolic and Molecular Bases of Inherited Disease*, eds, Scriver, C. R., Beaudet, A. L., Sly, W. S. & Valle, D. (McGraw–Hill), pp. 3733–3774.
6. Eng, C. M., Guffon, N., Wilcox, W. R., Germain, D. P., Lee, P., Waldek, S., Caplan, L., Linthorst, G. E. & Desnick, R. J. (2001) *N. Engl. J. Med.* **345**, 9–16.
7. Schiffmann, R., Kopp, J. B., Austin, H. A., III, Sabnis, S., Moore, D. F., Weibel, T., Balow, J. E. & Brady, R. O. (2001) *J. Am. Med. Assoc.* **285**, 2743–2749.
8. Kakkis, E. D., Muenzer, J., Tiller, G. E., Waber, L. Belmont, J., Passage, M., Izykowski, B., Phillips, J., Doroshow, R., Walot, I., *et al.* (2001) *N. Engl. J. Med.* **344**, 182–188.
9. Sly, W. S. (1993) *Nat. Genet.* **4**, 105–106.
10. Frisella, W. A., O'Connor, L. H., Vogler, C. A., Roberts, M., Walkley, S., Levy, B., Daly, T. M. & Sands, M. S. (2001) *Mol. Ther.* **3**, 351–358.
11. Ghodsi, A., Stein, C., Derksen, T., Martins, I., Anderson, R. D. & Davidson, B. L. (1999) *Exp. Neurol.* **160**, 109–116.
12. Ohashi, T., Watabe, K., Uehara, K., Sly, W. S., Vogler, C. & Eto, Y. (1997) *Proc. Natl. Acad. Sci. USA* **94**, 1287–1292.
13. Snyder, E. Y., Taylor, R. M. & Wolfe, J. H. (1995) *Nature (London)* **374**, 367–370.
14. Watson, G. L., Sayles, J. N., Chen, C., Elliger, S. S., Elliger, C. A., Raju, N. R., Kurtzman, G. J. & Podsakoff, G. M. (1998) *Gene Ther.* **5**, 1642–1649.
15. Bosch, A., Perret, E., Desmaris, N. & Heard, J. M. (2000) *Mol. Ther.* **1**, 63–70.
16. Bosch, A., Perret, E., Desmaris, N., Trono, D. & Heard, J. M. (2000) *Hum. Gene Ther.* **20**, 1139–1150.
17. Xia, H., Mao, Q. & Davidson, B. L. (2001) *Nat. Biotechnol.* **19**, 640–644.

18. Sly, W. S., Quinton, B. A., McAlister, W. H. & Rimoin, D. L. (1973) *J. Pediatr.* **82,** 249–257.
19. Hall, C. W., Cantz, M. & Neufeld, E. F. (1973) *Arch. Biochem. Biophys.* **155,** 32–38.
20. Brot, F. E., Glaser, J. H., Roozen, K. J., Sly, W. S. & Stahl, P. D. (1974) *Biochem. Biophys. Res. Commun.* **57,** 1–8.
21. Kaplan, A., Achord, D. T. & Sly, W. S. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 2026–2030.
22. Achord, D. T., Brot, F. E., Bell, C. E. & Sly, W. S. (1978) *Cell* **15,** 269–278.
23. Birkenmeier, E. H., Davisson, M. T., Beamer, W. G., Ganschow, R. E., Vogler, C. A., Gwynn, B., Lyford, K. A., Maltais, L. M. & Wawrzyniak, C. J. (1989) *J. Clin. Invest.* **83,** 1258–1266.
24. Berry, C. L., Vogler, C., Galvin, N. J., Birkenmeier, E. H. & Sly, W. S. (1994) *Lab. Invest.* **71,** 438–445.
25. Chang, P. L., Lambert, D. T. & Pisa, M. A. (1993) *NeuroReport* **4,** 507–510.
26. O'Connor, L. H., Erway, L. C., Vogler, C. A., Sly, W. S., Nicholes, A., Grubb, J., Holmberg, S. W., Levy, B. & Sands, M. S. (1998) *J. Clin. Invest.* **101,** 1394–1400.
27. Birkenmeier, E. H., Barker, J. E., Vogler, C. A., Kyle, J. W., Sly, W. S., Gwynn, B., Levy, B. & Pegors, C. (1991) *Blood* **78,** 3081–3092.
28. Sands, M. S., Erway, L. C., Vogler, C., Sly, W. S. & Birkenmeier, E. H. (1995) *Blood* **86,** 2033–2040.
29. Sands, M. S., Vogler, C., Kyle, J. W., Grubb, J. H., Levy, B., Galvin, N., Sly, W. S. & Birkenmeier, E. H. (1994) *J. Clin. Invest.* **93,** 2324–2331.
30. Sands, M. S., Vogler, C., Torrey, A., Levy, B., Gwynn, B., Grubb, J., Sly, W. S. & Birkenmeier, E. H. (1997) *J. Clin. Invest.* **99,** 1596–1605.
31. Vogler, C., Sands, M. S., Levy, B., Galvin, N., Birkenmeier, E. H. & Sly, W. S. (1996) *Pediatr. Res.* **39,** 1050–1054.
32. Vogler, C., Levy, B., Galvin, N. J., Thorpe, C., Sands, M. S., Barker, J. E., Baty, J., Birkenmeier, E. H. & Sly, W. S. (1999) *Pediatr. Res.* **45,** 838–844.

1: Gene Ther. 2000 May; 7(9): 759-63.

Related Articles, Links

nature publishing group

Widespread gene transfection into the central nervous system of primates.

Hagihara Y, Saitoh Y, Kaneda Y, Kohmura E, Yoshimine T.

Department of Neurosurgery, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan.

We attempted in vivo gene transfection into the central nervous system (CNS) of non-human primates using the hemagglutinating virus of Japan (HVJ)-AVE liposome, a newly constructed anionic type liposome with a lipid composition similar to that of HIV envelopes and coated by the fusogenic envelope proteins of inactivated HVJ. HVJ-AVE liposomes containing the lacZ gene were applied intrathecally through the cisterna magna of Japanese macaques. Widespread transgene expression was observed mainly in the neurons. The lacZ gene was highly expressed in the medial temporal lobe, brainstem, Purkinje cells of cerebellar vermis and upper cervical cord (29.0 to 59.4% of neurons). Intrastriatal injection of an HVJ-AVE liposome-lacZ complex made a focal transfection around the injection sites up to 15 mm. We conclude that the infusion of HVJ-AVE liposomes into the cerebrospinal fluid (CSF) space is applicable for widespread gene delivery into the CNS of large animals. Gene Therapy (2000) 7, 759-763.

PMID: 10822302 [PubMed - indexed for MEDLINE]

Display Abstract Show: 20 Sort Send to Text

1: Hum Gene Ther. 2002 Mar 20; 13(5): 665-74.

Highly efficient and specific gene transfer to Purkinje cells in vivo using a herpes simplex virus I amplicon.

Agudo M, Trejo JL, Lim F, Avila J, Torres-Aleman I, Diaz-Nido J, Wandosell F.

Centro de Biologia Molecular Severo Ochoa CSIC-UAM, Universidad Autonoma de Madrid, Madrid 28049, Spain.

The transduction of cerebellar neurons in vivo with herpes simplex virus 1 (HSV-1) amplicon carrying the lacZ gene has been investigated after injection of the vector in the cerebellar cortex, ventricles, and inferior olive of adult rats. Injection into the cerebellar cortex resulted in transduction of Purkinje cells near the needle tract and injection into the ventricles yielded no transduced neurons. In contrast, high transduction efficiency was achieved by vector injection into the inferior olive, resulting in one of three positive Purkinje cells all over the ipsilateral and contralateral cerebellar hemispheres. Because neurons in the deep cerebellar nuclei are also transduced, we suggest that the vector is delivered from the inferior olive to the cerebellar nuclei and then to Purkinje cells by retrograde axonal transport. Expression of the lacZ gene within Purkinje cells was surprisingly persistent and was maintained at the same level for at least 40 days. Importantly, no signs of either toxicity or inflammation were observed in the cerebellum after vector injection, except for the borders of the needle tract where some reactive astrocytes were detected. Indeed, motor coordination of treated animals was entirely normal, as assessed by the rota-rod test. These results demonstrate that HSV-1 amplicon vectors can effect safe and stable transgene expression in Purkinje cells in vivo, raising the possibility of using these vectors for long-term gene therapy of human cerebellar disorders.

PMID: 11916489 [PubMed - indexed for MEDLINE]

1: Brain Res Mol Brain Res. 2003 Nov 6; 119(1): 1-9.

Related Articles, Links

ELSEVIER FULL-TEXT ARTICLE

Systemic FIV vector administration: transduction of CNS immune cells and Purkinje neurons.

Kyrkanides S, Miller JH, Federoff HJ.

Department of Dentistry, School of Medicine and Dentistry, University of Rochester, Rochester, NY, USA. stephanos_Kyrkanides@urmc.rochester.edu

The systemic effects of gene therapy have been previously described in a variety of peripheral organs following intravenous administration or intraperitoneal inoculation of viral vectors, as well as in the brain following intracranial administration. However, limited information is available on the ability of viral vectors to cross the blood-brain barrier and infect cells located within the central nervous system (CNS). We employed a VSV-G pseudotyped FIV(lacZ) vector capable of transducing dividing, growth-arrested, as well as post-mitotic cells with the reporter gene lacZ. Adult mice were injected intraperitoneally with FIV(lacZ), and the expression of beta-galactosidase was studied 5 weeks following treatment in the brain, liver, spleen and kidney by X-gal histochemistry and immunocytochemistry. Interestingly, relatively low doses of FIV(lacZ) administered intraperitoneally lead to beta-galactosidase detection in the brain and cerebellum. The identity of these cells was confirmed by double immunofluorescence, and included CD31-, CD3- and CD11b-positive cells. Fluorescent microspheres co-injected with FIV(lacZ) virus were identified within mononuclear cells in the brain parenchyma, suggesting infiltration of peripheral immune cells in the CNS. Cerebellar Purkinje neurons were also transduced in all adult-injected mice. Our observations indicate that relatively low doses of FIV(lacZ) administered intraperitoneally resulted in the transduction of immune cells in the brain, as well as a specific subset of cerebellar neurons.

PMID: 14597224 [PubMed - in process]

Display Abstract Show: 20 Sort Send to Text